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Angiogenesis is critical for tumor growth and metastasis. The relationship between the degree of tumor angiogenesis and poor outcome has been reported. Little is known about the regulation of this process. Our general hypothesis is that tumors behave like wounds that heal in an abnormal fashion. Angiogenesis is a normal component of wound healing. Thus, we investigated whether cytokines that regulate wound healing would also be found in human breast cancer.

Interleukin 8 (IL-8) is an inflammatory cytokine that is also a known angiogenic factor. The goals of this project were to 1) establish the presence of IL-8 in human breast cancer samples; 2) investigate whether human breast cancer cells produce IL-8 in an *in vitro* model and dissect the factors that regulate that process; and 3) develop an *in vivo* model of human breast cancer in immunodeficient model and attempt to block angiogenesis generally and IL-8 specifically in order to slow tumor growth. The first two goals have been met, the third has not.

Much of the data generated has been presented in earlier Annual Reports. Other data has been published, presented or is in manuscript.

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Annual Report Body

Introduction

This Final Report will focus on the overall goals and objectives of this project as originally described. Many of the results have been presented in previous Annual Reports, or published. These will not be repeated in this document. Areas where further progress was made will be reported, or published literature appended. We plan on continuing to analyze the data that has been obtained. Those areas will be described.

The primary hypothesis of this project was that cancers behave like wounds that heal in an abnormal fashion [1] [2]. Like wounds, cancers rely on nutrients to grow. In order to obtain these nutrients, cancers and wounds produce signals that recruit support. While wounds stop signaling once they are healed, cancers continue to recruit by producing, or inducing the production of factors such as cytokines. One of the important requirements for tumor growth is the ingrowth of blood vessels in order to supply oxygen and nutrients. This process known as angiogenesis has emerged as a key factor in tumor growth and metastasis [3-7].

In the last decade a large number of such angiogenic factors (AF) has been described, and the number of known or suspected factors has continued to increase [8-12] In our laboratory, we have done extensive work on one established AF, interleukin 8 (IL-8) [13-20]. We have studied IL-8 in head and neck squamous cancer [21-26], colon cancer and prostate cancer [27, 28].

In order to demonstrate the importance of IL-8 in breast cancer, described three Spesific Aims. First, IL-8 should be found in human breast cancer specimens. Second, IL-8 must be produced by, or induced by human breast cancer cells. Third, inhibition of IL-8 should interfere with the growth of breast cancer tumors in an animal model. In addition, we investigated factors that are involved in the regulation of IL-8 expression. In order to investigate our central hypothesis, we chose the regulatory cytokines that are involved in wound healing.

Background

Breast Cancer: Many factors beyond tumor size, histologic grade, and lymph node involvement have been examined as prognostic indicators for metastatic breast disease [29]. Recently, a

considerable body of evidence has demonstrated that in invasive breast cancer, angiogenesis is associated with poor outcome as demonstrated by a shorter disease free survival and an increased frequency of metastatic disease [30]. Furthermore, the degree of intratumoral vascularization has also been shown to be a significant and independent prognostic factor in primary breast cancer [4, 31, 32]. It is also well accepted that tumors can not grow beyond several millimeters in diameter without recruiting new blood vessels. Thus, the control of neovascularization is critical to the growth and spread of malignancies.

A second key component in the growth and spread of human breast cancer (HBC) is cell proliferation, which is modulated by autocrine and/or paracrine factors [33, 34], in particular, cytokines (*e.g.* growth factors and angiogenesis factors). Virtually all nucleated cells including breast cells, can be induced to express cytokines *e.g.*, tumor necrosis factor (TNF) [35], interleukin (IL)1 [36], IL3 [37], and IL6 [38, 39]. Recently, the cytokine IL8 has been demonstrated to be: ***a***) an autocrine tumor cell growth factor [40], ***b***) a potent vascular endothelial cell migration factor *in vitro* [41], ***c***) able to induce neovascularization in the rat cornea [42], ***d***) correlated with melanoma metastatic potential in the nude mouse model [43], and ***e***) a predictive indicator of therapeutic response and prognosis of patients with recurrent breast cancer [44]. IL8 receptors are expressed on both breast tumor and vascular endothelial cells from patient tissue samples [45]. This data suggests that tumor derived IL8 could contribute to tumor growth directly by autocrine stimulation of tumor cell proliferation, and indirectly, by paracrine modulation of vascular endothelial cells leading to neovascularization of breast cancer which subsequently contributes to growth and metastasis.

Interleukin-8 (IL8): IL8 is a member of a family of 8-10 kD cytokines that are involved in proinflammatory and reparative processes. Cytokines in this family are basic heparin-binding proteins that display chemotactic activities *in vitro* and *in vivo* [46]. The presence of IL8 has been extensively described in two inflammatory conditions (i.e. psoriasis and rheumatoid arthritis). These diseases are characterized by exacerbation/remission cycles, host cellular influx and host induced tissue damage. Distinct from other cytokines, *e.g.* IL2 and IFN which are produced by specific cell types, IL8 has been shown to be produced by several cell types including leukocytes, monocytes/macrophages, lymphocytes, fibroblasts, endothelial cells, smooth muscle cells, chondrocytes, keratinocytes, and epithelial cells in response to IL1 and TNF stimulation [46]. IL8

expression has been demonstrated in tumor cell lines including carcinoma and sarcoma cells. Regulation of IL8 production occurs at both the pre and post-transcriptional levels and varies with the cell type analyzed. With IL1 induction, IL8 transcripts can be detected as early as 0.5 hrs and maintained for as little as three hours, as with monocytes [47] or expressed at high levels for as long as sixteen hours with fibroblast and endothelial cells [48]. The entire IL8 gene has been cloned and sequenced [49]. Interestingly, the 5 prime flanking region of IL8 gene contains several potential binding sites (enhancer/promoter) for known nuclear factors, which explains the induction of IL8 transcripts and protein by IL1 and TNF.

The IL8 Receptors (IL8RA and IL8RB): IL8 receptors are present on a variety of cell types. Neutrophils, monocytes, keratinocytes, endothelial cells, fibroblasts, macrophages, smooth muscle and T cells have all been shown to express one or both of these receptors. To date, two distinct IL8 receptors have been isolated, characterized and cloned. These receptors are 77% homologous and contain seven transmembrane domains. Both receptors act *via* G proteins through the phospholipase C pathway which induces the release of intracellular calcium and the activation of protein kinase C [46]. Both IL8RA and IL8RB bind IL8 with high affinity (Kd = 0.1-4.0 nM). Several other cytokines also bind to only IL8RB with high affinity. Neutrophil activating protein (NAP-2) and melanoma growth stimulatory activity (MGSA, also known as GRO) bind IL8RB with affinity similar to IL8, but show a much lower affinity for IL8RA (Kd=100-130 nM) [50]. Therefore, while IL8RA is specific for IL8, IL8RB binds a variety of compounds. A third IL8 receptor, know as the Duffy Antigen/Receptor for Cytokine (DARC), first identified on erythrocytes, functions not only as a promiscuous chemokine receptor, but also as a receptor for the parasite *Plasmodium vivax* [51]. DARC has been shown to bind IL8 with high affinity (Kd=20+/- 4.7nM) for DARC transfected K562 cells [51] and (Kd=9.5+/- 3.6nM) for erythrocyte ghosts [52]. It's function on erythrocytes is postulated to act as a "sink" or clearance factor for chemokines, limiting their availability in the circulation, and thereby regulating inflammation. DARC has also been shown to be expressed on post capillary venue endothelial cells [53, 54]and although it will internalize radiolabeled ligand [54], no signal transduction function has been described.

SPECIFIC AIM I: *TO CHARACTERIZE IL-8 EXPRESSION IN HUMAN BREAST CANCER*

Task 1 was accomplished in the first two years of this project. We established the presence of IL-8 in human breast cancer specimens. The results of these studies have been presented in previous Annual Reports, no further work has been done in these areas.

Study IA- To characterize IL-8 antigen distribution in human breast biopsy specimens

We have not added any new specimens to this Study analysis. Our early studies clearly demonstrated the presence of IL-8 antigen in human breast cancer specimens. IL-8 was found more often in specimens from patients with invasive ductal cancer (IDC), less frequently in specimens from patients with ductal carcinoma *in situ* (DCIS), and only occasionally in patients with benign breast disease [55].

If IL-8 is functioning to promote angiogenesis in the tumors, than patients whose tumors contain more IL-8 antigen might have a worse clinical outcome. In order to demonstrate this relationship, we need to obtain follow-up data on the subjects in this study. The original project had IRB approval to obtain anonymized tissues. We are in the process of obtaining IRB approval to obtain clinical outcome information in order to carry out these correlations.

Study IB- To demonstrate IL-8 mRNA expression in human breast cancer tissues

This Study was not carried out. Our *in vitro* data demonstrated that breast cancer cells (BCC) were capable of producing IL-8. We therefore dropped this particular study for reasons of budget and time expenditure that would have been needed.

Study IC- To Correlate IL-8 expression with neovascularization in human breast biopsy specimens

As reported in previous Annual Reports, we spent a considerable amount of time and effort attempting to quantify blood vessels in the breast specimens. We used the two accepted stains for blood vessels, Factor VIII and anti CD-31. We found the vessel count results to be unreliable. The number of vessels did not correlate with the invasiveness of the tumors. Interestingly, despite

initial enthusiasm for this approach in the literature [3, 4, 56, 57], in the past few years, more recent articles have questioned the reliability of this technique [58, 59].

Other Studies on Human Breast Cancer Specimens

As a result of our *in vitro* studies described below, we determined that a number of regulatory cytokines played a role in IL-8 production. Accordingly, we investigated the presence of these cytokines and their receptors in the breast cancer specimens. The results of these studies were published or presented at meetings, and are attached in the Appendix [45, 60]. We also examined the relationship between IL-8 expression and estrogen and progesterone receptors. These studies demonstrated that patients whose tumors demonstrated higher levels of IL-8 were more likely to be estrogen and progesterone receptor negative, a surrogate marker of poor outcome [61].

SPECIFIC AIM II: *To characterize IL-8 expression by breast cancer cell lines in vitro*

Having demonstrated the presence of IL-8 in human breast cancer specimens, it was necessary to determine the source of the IL-8. Several cells might have been the source of the cytokine, including breast tumor cells, normal breast cells, endothelial cells, or infiltrating blood cells. It was our hypothesis that the breast cancer cells were either the source of the IL-8, or produced a stimulatory cytokine, inducing IL-8 secretion by native cells. Regardless of the source, angiogenesis would be promoted supporting tumor growth and metastasis.

Study IIA- To characterize the expression of IL-8 antigen by cultured human breast cancer cell lines

This study was completed in Year 03, and reported in previous Annual Reports. We have been unsuccessful in getting it published.

Study IIB- To quantify IL-8 expression in cytokine stimulated breast cancer cells

Having established that IL-8 was associated with breast cancer cells in human tumors (Specific Aim I), and that breast cancer cells could produce IL-8 *in vitro*, we next sought to investigate the factors that regulated the expression of IL-8 by the breast tumor cells. Accordingly, cultured breast cancer cells were exposed to a variety of cytokines known to regulate normal wound healing and angiogenesis. The results of these experiments were previously reported in our

Annual Reports, but the manuscript is not yet published (see Appendix).

The results of Studies A and B clearly showed that human breast cancer cells can produce IL-8, and that this production is regulated by cytokines. The expression of IL-8 was related to estrogen sensitivity of the cell lines. The regulatory cytokines were similar to regulatory cytokines seen in wound healing.

Study IIC- To quantify IL-8 expression in co cultures of MCF-7 and BT-20 cells

Due to a lack of time and resources, this Study was not completed. In retrospect, we do not feel that meaningful information would be gained.

SPECIFIC AIM III- TO CHARACTERIZE IL-8 ANTIGEN EXPRESSION AND NEOVASCULARIZATION IN HUMAN BREAST CANCER CELLS GROWN AS TUMORS IN NUDE MICE

Study IIIA: To demonstrate IL-8 antigen expression in subcutaneously implanted human breast cancers

Introduction and Rationale: Our original hypothesis was that IL-8 is an important angiogenic factor regulating neovascularization and tumor growth in human breast cancer. Our earlier *in vitro* and immunohistochemical studies of human breast tissue clearly demonstrated that human breast cancer cells are capable of producing IL-8, and that IL-8 is found in association with breast cancer cells in human tumors. Based on these observations, we next sought to establish the presence and distribution of IL-8 in an *in vivo* model of human breast cancer in immunodeficient mice. This step was fundamental to our planned experiments to manipulate the expression of angiogenic factors in the mice in order to control tumor growth.

Obstacles: In order to study the expression and growth of tumors *in vivo*, we selected tumor cell lines based on our *in vitro* data, with high (MDA-MB-231), medium (ZR-75) and low (MCF-7) IL-8 expression. Our early attempts at growing breast tumor cell lines in immunodeficient mice demonstrated highly variable success rates, usually less than 50% success in tumor growth. We reviewed the literature exhaustively and called several prominent investigators working in the area of human mouse xenografts. Through these efforts we discovered that this low success rate for

human –mouse xenografts was not unusual. However, we felt that improvements could be made to better guarantee obtaining consistent tumor growth for our studies using angiogenesis inhibitors (see below). To that end we have adapted the techniques used by other laboratories to enhance tumor growth and reproducibility in the immunodeficient mice. Specifically, we have incorporated Matrigel® into the tumor cell- media solutions used for injection of the tumor cells into the mice. The use of this matrix increased our yield of tumor growth to almost 100%. Matrigel is the tumor matrix derived from mouse tumor cells grown *in vitro*, and is known to promote tumor growth in vitro due to the presence of both basement membrane, and a large number of tumor growth factors. Thus we hypothesize that the Matrigel provides a matrix that allow the tumor cells to initially adhere as well as provide numerous growth factors all of which provide a favorable environment for growth of the tumors in the early stage. Overcoming this obstacle significantly delayed the completion of this project but it was essential to develop a reliable tumor model for our inhibitor studies. Once this was overcome, the Studies outlined could proceed.

Results: Immunohistochemical analysis of the xenograft human breast cancer tumors indicated that all three tumor cell lines expressed IL-8 antigen when grown *in vivo*. Additionally, immunohistochemical analysis of these same tumors for IL-8 receptor also indicated that they expressed these receptors *in vivo*. Interestingly we saw that there was a differential expression of the IL-8 receptors on the various HBC tumor cells (see table 1). These observations are consistent with our previous *in vitro* and human breast cancer specimen observations.

TABLE 1: SUMMARY OF TUMOR ASSOCIATED IL-8/IL-8R IMMUNOHISTOCHEMICAL STAIN PROFILES IN HBC-MOUSE XENOGRAFT TISSUE

HBC CELL LINES	IL-8	IL-8 RA	IL-8 RB
MDA-MB-231	+++	-	+
ZR-75	++++	+	+/-
MCF-7	+	-	+/-

1+=faint; 2+=definate; 3+=moderate; 4+=strong

We then analyzed homogenates of the explanted xenograft tumors for IL-8 levels by ELISA. The results of this analysis confirmed the expression of IL-8 by breast tumors *in vivo*. The values obtained paralleled our observations from our *in vitro* experiments described in previous Annual Reports , i.e. high IL-8 producers *in vitro* were also high IL-8 expressers *in vivo*, and low producers in vitro tended to be low producers in vivo. Currently there are no quantitative ELISA analyses available for the IL-8 receptors; thus quantitation of IL-8 receptors could not be done.

TABLE 2: IL-8 EXPRESSION IN XENOGRAFT MODEL COMPARED TO IL-8 EXPRESSION IN *IN VITRO* CELL CULTURE

HBC CELL LINES	IL-8 levels in vitro (pg/ml (IL-1ß stimulation)	IL-8 levels In vivo (pg/mg protein)
MDA-MB231(high IL-8 expresser)	356,960 ± 61100	34.0; 9.3; 16.0 (n=3)
ZR-75 (mod.IL-8-expressors)	32,600 ± 4080	N.D.
MCF-7 (low IL-8 expresser)	40 ± 13	10.4; not detectable (n=2)

Interpretation: The limited number of mice included in this study is a reflection of the difficulty we encountered in setting up this model. However, the *in vivo* results parallel the previous in vitro data we reported (39,40). The tumor cells maintain their ability to express IL-8 when grown in immunodeficient mice. The cell lines that were high and moderate expressers of IL-8 *in vitro* were higher expressers of IL-8 *in vivo*. These observations support the use of this model in later experiments designed to manipulate angiogenic factors.

Future Plans: This experiment formed the foundation for the studies described below. Our approach was to 1) specifically block IL-8 with monoclonal antibodies, and 2) non-specifically inhibit angiogenesis using a known angiogenesis inhibitor (see below).

Study IIIB: To establish a dual tumor model using MCF-7 and BT-20 cells

Our success in growing the tumors, and identification of IL-8 in the mouse tumors led us to experiments involving anti-angiogenic agents. For that reason it was decided to pursue those studies and postpone or eliminate this study.

Study IIIC: The role of angiogenesis inhibitors

Experiment 1: Tumor growth rates

Introduction and rationale: In order to study the effects of angiogenesis inhibitors on implanted tumors, we first needed to establish the baseline rates of growth of the tumors under standard conditions. We selected the same cell lines used in the previous study. Our approach was to characterize the growth of both a high and low expressers of IL-8 in the xenograft model. If our hypothesis was correct, that IL-8 is an important factor in tumor growth and angiogenesis, then tumor growth by AF blockers or inhibitors, anti IL-8 or thalidomide, would be greatest in the IL-8 producing tumors. Further, the relative inhibition of tumor growth would be greatest in the tumors that expressed the highest level of IL-8.

Obstacles: Similar difficulties were encountered in these experiments that were conducted simultaneously with studies IIIa. Having established the optimal conditions for growing HBC cell lines in immunodeficient mice, allowed us to obtain the preliminary data presented.

Methods: The same cell lines used for tumor growth kinetic studies were used in this experiment. Briefly, 1 X 10^7 tumor cells were suspended in Matrigel and injected into the mammary pads of the mice.

Results: Our growth studies indicated that HBC cell lines that were high IL-8 expressers grew more rapidly then did the moderate or low IL-8 expressing strains in our xenograft model (see Figure 1)

Interpretation: This data demonstrates that there is variation in tumor growth in the xenograft model and that the growth correlates with the general levels of IL-8 expression in vitro and in vivo, supports our hypothesis on the role of tumor cell derived IL-8 in HBC growth.

Future Plans: *The* number of vessels identified in the tumors will be counted. This will allow us to compare the rate of angiogenesis in the tumors tested. If our hypothesis regarding IL-8 is correct, higher levels of angiogenesis will be found in the tumors from HBC that are high expressers of IL-8.

Experiment 2: Anti-IL-8 antibody injection

Introduction and rationale: Our central hypothesis is that IL-8 is an important AF and growth factor for human tumors. The experiments described above established that HBC express IL-8 *in vivo*, and that the tumors grown in immunodeficient mice express receptors for IL-8. The

next phase of this study was to determine if the addition of anti-IL-8 antibody to the mice would inhibit tumor growth.

Methods: The same cell lines used for tumor growth kinetic studies were used in this experiment. Briefly, 1 X 10^7 tumor cells were suspended in Matrigel and injected into the mammary pads of the mice. Two injection sites were used per mouse. Anti IL-8 antibody was prepared as previously described [62]. The antibody was prepared in PBS at a concentration of 10 mg/ml. Beginning on Day -1, relative to HBC cell line injection, 0.5 ml of antibody was injected i.p into the mice. Control IgG was prepared and injected i.p. at the same schedule as the specific antibody. Tumor growth was measured using calipers twice a week. After 5 weeks, the mice were sacrificed, and the tumor harvested. The tumors were cleared of surrounding tissue and weighed.

Obstacles: Significant problems were encountered in repeated i.p. injections in the mice two times a week. On sacrifice of the mice, multiple adhesions, and evidence of peritonitis and adhesions/fibrosis was seen. The inflammation/adhesions/fibrosis, seen in response to the repeated i.p. injections, likely resulted in erratic absorption of the antibody from the peritoneal cavity. We also postulate that after repeated injections, immune complexes may develop at tumor sites that could mediate macrophage/PMN mediated ADCC reactions. These obstacles can easily account for the results described below.

Results: Tumor growth is plotted as size in mm^2 in Figure 2A and 2B. Early results seemed to indicate inhibition of tumor growth when compared to the control IgG injected mice. Unfortunately, these results did not persist. Additionally the decrease in tumor size was likely to the extensive necrosis that occurred in the later stages of tumor growth.

Interpretation: The early inhibition of tumor growth appeared quite promising. The anti IL-8 Ab may have been effectively blocking tumor growth in the early stage. Our observation regarding the development of adhesions and inflammation in the peritoneum of the mice can also account for the variability in tumor growth seen. As time progressed, the number of IL-8 receptors may have increased, or the amount of IL-8 produced by the tumor cells may have increased. The net result would be a need for increasing doses of anti IL-8 antibody.

Future Plans: For the above-mentioned reasons, it became evident that theoretic as well as practical considerations dictated that we not continue these studies at this time. Instead attention was paid to a non-specific inhibitor of tumor angiogenesis, thalidomide.

Experiment 3: The role of thalidomide on tumor growth

Introduction and rationale: Despite the difficulties encountered on Experiment 2, the rationale for inhibiting tumor growth by blocking angiogenesis remains valid. Thalidomide has been shown to inhibit angiogenesis when given orally inflammatory models of neovascularization. However in our xenograft model, gavage feeding is impractical. Likewise, simple addition of thalidomide to the drinking water of the mice would have resulted in unpredictable dosing, due to multiple animal housing and spillage. For these reasons, we elected to prepare the drug in absorbable pellets that could be implanted subcutaneously. The rationale was that this approach would allow for consistent, accurate drug release.

Obstacles: Technology for consistent delivery of thalidomide has not been developed thus we had to develop it. Thus we utilized pellets to give sustained drug release. Short-term high burst of thalidomide possibly accounted for early encouraging results, but with time local drug levels were lost. Clearly a more sustained drug release system will need to be developed

Results: Encouraging early results, suggest that thalidomide may be a potential useful inhibitor of angiogenesis, but a more sustained drug delivery system must be developed.

Future Plans: in future studies we hope to use PEG biodegradable beads as a better thalidomide delivery system to test thalidomide's usefulness as an anti angiogenic drug.

Specific Aim IV-Additional Studies

Study IVA: Interleukin 1 family and Breast Cancer

Previous studies have demonstrated the key role of the IL-1 family of cytokines and receptors in a wide number of immunologic and inflammatory diseases, but little is known about the existence and role of these cytokines and their receptors in human cancers, including human breast cancer. The importance of IL-1 family of cytokines and receptors in disease has lead us to develop the following hypothesis: 1) Human breast tumor cells express the IL-1 family of cytokines (IL-1a,

IL-1B and IL-1RA) and receptors (IL-1RI and IL-1RII) and; 2) the local expression of the IL-1 family of cytokines and receptors within the tumor microenvironment can control tumor expression of protumorogenic cytokines such as IL-8. To begin to test this hypothesis we characterized the in vivo and in vitro expression of IL-1a, IL-1B, IL-1RA as well as the IL-1 receptors RI and RII by human breast cancer cells. To initially demonstrate the presence and the distribution of IL-1 cytokines and receptors, as well as IL-8 in human breast disease, archival specimens from 7 benign, 8 DCIS, and 25 invasive human breast tumors were analyzed using standard immunohistochemical techniques. Immunohistochemical studies demonstrated that IL-1a, IL-1B, IL-1RA as well as IL-1RI and IL-1 RII were expressed on both DCIS and invasive tumor cells. Additionally, IL-1RI receptors appear to be expressed at higher levels in invasive breast tumor cells when compared to DCIS tumor cells and benign breast disease. Interestingly, vascular endothelial cells, fibroblasts and smooth muscle cells in the tumor microenvironment also expressed IL-1 receptors. We next determined the HBC tissue levels and correlation's of the IL-1 and IL-8 cytokines, using ELISA technology. Quantitative studies of the tumor homogenates demonstrated that not only IL-1a and IL-1B were present in the tumor tissue, but that IL-8 was also present. Additionally we demonstrated that IL-1a and IL-1B levels correlated directly with IL-8 levels in HBC tissue. Parallel studies using these same immunoassays on tumor tissue from tumor lines (MCF 7, ZR 75, and MDA) grown in a nude mouse xenograft model of HBC, indicated that the tumor cells also expressed the IL-1 family of cytokines and receptors and IL-8 in vivo. To directly demonstrate the ability of these HBC cell lines to express IL-1 and IL-8 cytokines we initiated in vitro studies. Our in vitro studies demonstrated that human breast cancer cell lines (MCF 7, ZR 75 and MDA) not only expressed IL-1a, IL-1B IL-1RA, IL-1RI and IL-1 RII, but that these tumors cells can be induced by IL-1a or IL-1B to express the protumorgenic cytokine IL-8. These data clearly demonstrate the presence and distribution of IL-1 cytokines and receptors in HBC, and suggest that the local expression of IL-1 by tumor cells likely results in the activation of a number of cells in the tumor microenvironment, productive of the expression of numerous protumorgenic activities such as IL-8 which would induce angiogenesis, tumor proliferation, and tumor invasion. These studies also suggest that targeting of the IL-1RI receptor may provide new approaches in HBC therapy.

Summary

Overall, we feel that we have met most of the goals in the original research proposal. Our hypothesis was that breast cancers behave like wounds that heal in an abnormal manner. Specifically, we postulated that breast cancer growth would respond to inflammatory cytokines, including those normally associated with wound healing and inflammation. One important aspect of wound healing that has been demonstrated to correlate with outcome in breast cancer is angiogenesis.

Since IL-8 was known to be such an inflammatory cytokine, and an angiogenic factor, it was reasonable to study it in breast cancer. Our early data clearly showed that IL-8 was associated with human breast cancer in breast cancer samples. In support of this model, we also demonstrated that the receptors for IL-8 were also found in the breast cancer specimens. Analysis of breast tumor homogenates further showed that IL-8 was present in breast cancer specimens, and correlated with a known indicator of poor outcome, lack of estrogen receptors.

Our *in vitro* studies examined the ability of cultured breast cancer cells to produce IL-8. We found that a variety of breast cancer cells produce IL-8 and that this production is regulated by IL-1 and TNF. This data further supported our hypothesis that the breast tumors are responding in an abnormal manner to inflammatory cytokines.

Our *in vivo* model was only moderately successful. Our initial attempts to establish tumor growth proved more difficult than expected. Once we were successful in growing the tumors in the nude mice, we were able to identify IL-8 expression by the tumor cells. The next and final phase of the studies was to try and inhibit tumor growth by blocking angiogenesis. Specifically, we attempted to block IL-8 using IL-8 antibodies, and we used implanted pellets of thalidomide. Further attempts to refine our techniques were unsuccesful.

Thus, we have shown that IL-8 is present in human breast cancer, levels of IL-8 correspond to other markers of outcome, that breast cancer cells can produce IL-8, and this production is under the control of cytokines such as IL-1 and TNF. This data supprots our overall hypothesis. In

future studies, we will be examining knockout of IL-8 to try and prevent angiogenesis and tumor growth.

Original Statement of Work

Task 1 Year 1 - To characterize IL-8 expression in human breast cancer

Study IA- To characterize IL-8 antigen distribution in human breast biopsy specimens

Study IB- To demonstrate IL-8 mRNA expression in human breast cancer tissues

Study IC- To Correlate IL-8 expression with neovascularization in human breast biopsy specimens

Task 2 Year 2- To characterize IL-8 expression by breast cancer cell lines in vitro

Study A- To characterize the expression of IL-8 antigen by cultured human breast cancer cell lines

Study B- To quantify IL-8 expression in cytokine stimulated breast cancer cells

Study C- To quantify IL-B expression in co cultures of MCF-7 and BT-20 cells

Task 3. Years 3-4- To characterize IL-8 antigen expression and neovascularization in human breast cancer cells grown as tumors in nude mice

Study A- To demonstrate IL-8 antigen expression in subcutaneously implanted human breast cancers

Study B- To establish a dual tumor model using MCF-7 and BT-20 cells

This Statement of Work has been modified to reflect 75% of the original budget. The final study has been omitted due to the lack of resources. Based on results from Year 03, the *in vivo* portion of this study will likely be modified

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FIGURE 1

GROWTH CURVES FOR AB CONTROLS FOR THREE CELL LINES

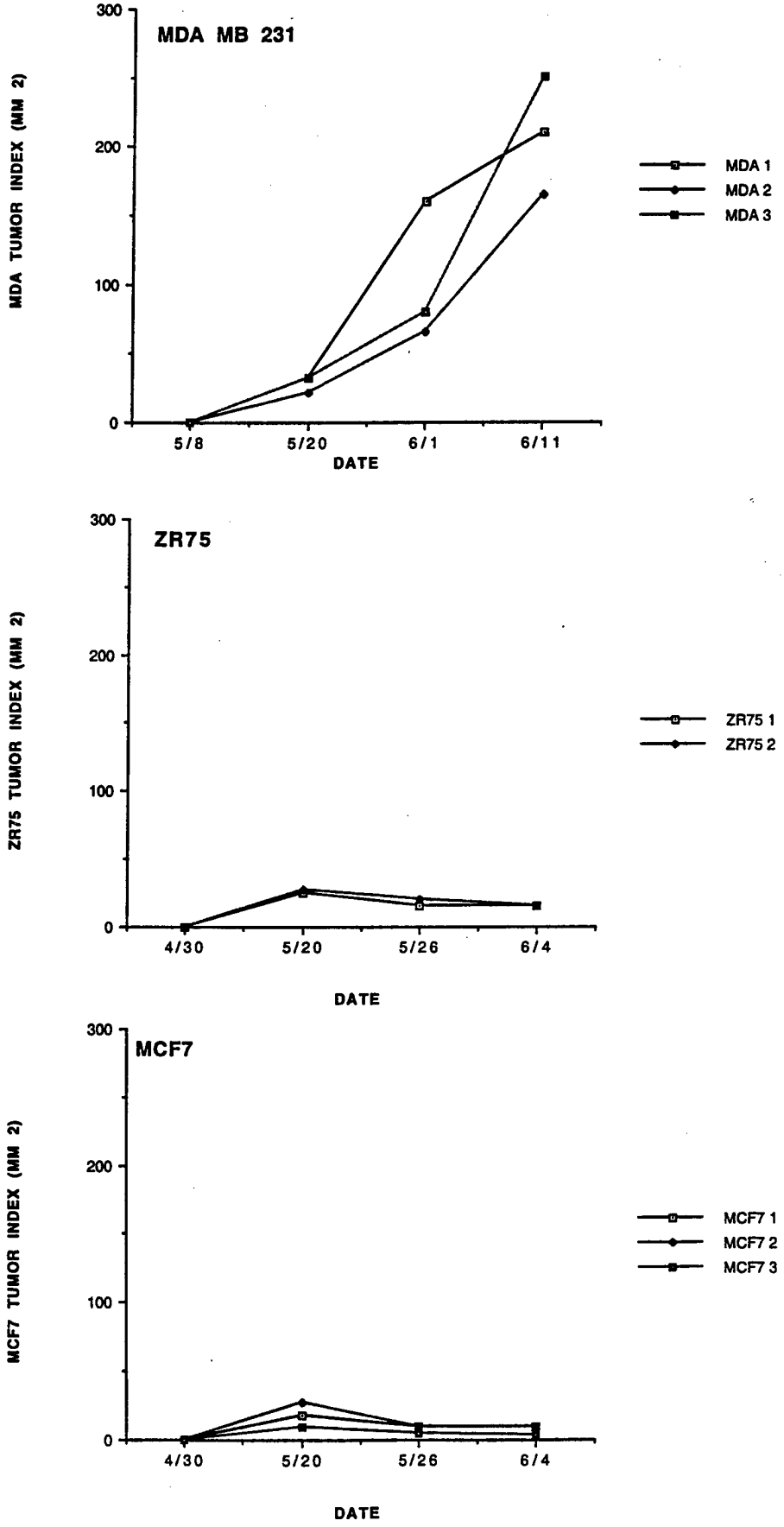
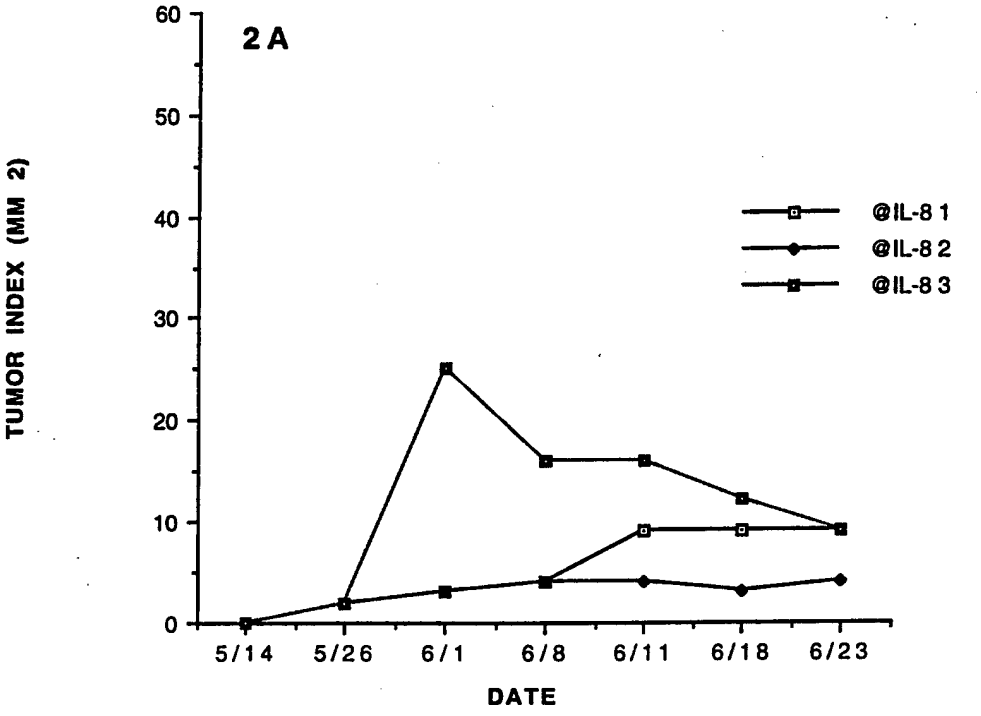
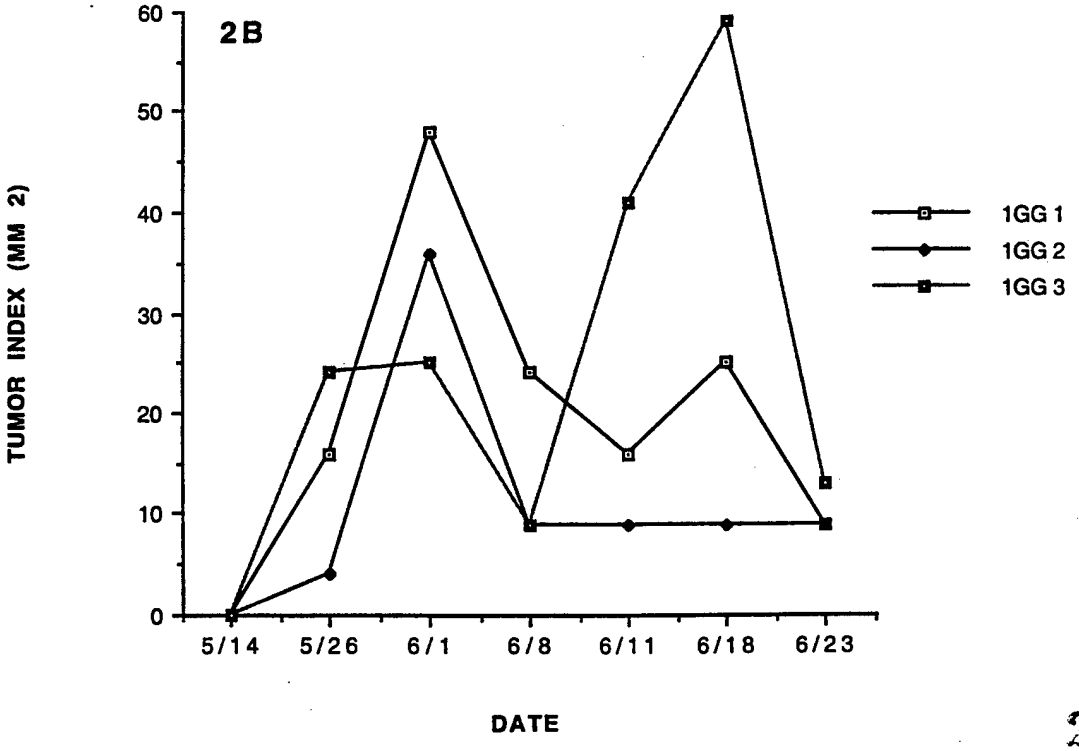


FIGURE 2

ZR-75 TUMOR GROWTH IN @IL-8 INJECTED MICE



ZR-75 TUMOR GROWTH IN IGG INJECTED MICE



Personnel Who Received Salary Support
(both are lab technicians)

1. Kathleen Anderson
2. Lauri Miller

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INCREASED ANGIOGENESIS FACTOR EXPRESSION IS ASSOCIATED WITH NEGATIVE HORMONE RECEPTOR STATUS IN HUMAN BREAST CANCER

Scott H. Kurtzman, MD, FACS, Kathleen Anderson, BA,
Yanping Wang, MD, Lauri Miller, BS,
George H. Barrows, MD, and Donald L. Kreutzer, PhD

ANGIOGENESIS IS CRITICAL for breast tumor growth and metastasis. This process is regulated by a number of chemical signals known as angiogenic factors (AF).[1] One recently identified AF is designated interleukin-8 (IL-8).[2] Although other AF have been implicated in cancer, previously nothing was known regarding the existence and importance of IL-8 in tumor cells in human breast cancer. Recently, we have demonstrated by immunohistochemical methods the presence of IL-8 in the tumor cells found in specimens of human breast cancer.[3]

Previous studies have correlated high levels of tumor angiogenesis with poor outcome in breast cancer patients.[4] Because breast tumors that exhibit negative hormone receptor status are also associated with poor outcome, we hypothesized that the levels of IL-8 in breast tumor tissue homogenates would inversely correlate with estrogen and progesterone receptor levels (ER/PR) in the tissue homogenates. Specifically, specimens with high levels of IL-8 (increased angiogenesis) would be associated with other markers of poor outcome such as lack of ER/PR.

MATERIALS AND METHODS

For these studies, tissues obtained from 39 patients being operated on for breast cancer were sent for ER/PR level determination, which was measured by standard enzyme-linked immunosorbent assay (ELISA). Protein levels were determined in these specimens using the Bradford reagent, and the data are corrected to mg cytosol protein. ER/PR levels greater than 15 fg/mg cytosol protein were considered positive. The tissue homogenates that were prepared for ER/PR analysis were then assayed for IL-8 using standard radioimmunoassay (RIA) technology. The IL-8 levels are expressed in pg/mg cytosol protein. Levels greater than 10 ng/mg cytosol protein were considered positive.

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Correlation of high and low IL-8 responders with ER/PR status in breast cancer homogenates

IL-8 responders: pg/mg protein (range)	ER negative/total	PR negative/total
Low <10 (1.9–10)	3/15 (20%)	5/15 (33.3%)
High >10 (11.2–8,276)	11/24 (47.8%)	16/23 (69.6%)

RESULTS

Of the 39 breast cancer specimens, 14 (36%) were ER negative and 19 (49%) were PR negative. We found that there were two populations of breast cancers, low IL-8 responders (n = 15) and high IL-8 responders (n = 24) (see Table). Of the low IL-8 responders, only 20% were ER negative and only 33.3% were PR negative. Consistent with our hypothesis, the homogenates that had high IL-8 levels (>10 pg/mg cytosol protein) were associated with negative ER (47.8%) and PR (69.6%) status.

DISCUSSION

In order for tumors to grow more than a few millimeters in size, they must induce new blood vessel growth (angiogenesis). This process is regulated by angiogenic factors. In normal wound healing, angiogenic factors are produced by infiltrating leukocytes, endothelial cells, and tissue cells such as fibroblasts. We hypothesize that in the tumor microenvironment, AF are directly produced by tumor cells. This production of AF then supports tumor growth and metastasis in an autocrine manner. As a result, we would expect that tumors that are able to produce AF would be associated with a worse outcome. Because follow-up data were not available on the patients included in this study, we correlated the levels of IL-8 with an established marker of poor patient outcome, lack of ER/PR.

CONCLUSIONS

These data support our hypothesis that the poor outcome associated with negative hormone receptor status may be related to increased levels of IL-8 produced by the tumors. This IL-8 production results in increased angiogenesis, which supports tumor growth and metastasis. Further studies to elucidate the interrelationship between other AF expression (eg, VEGF, bFGF) in breast tumor homogenates, hormone receptors, and poor outcome are in progress. These very interesting initial studies also will need to be validated in a larger patient population and correlated with patient outcome data. In the future,

inhibition of angiogenic factors such as IL-8 or the cytokines that control them may be an important adjunct in the treatment of patients with breast cancer.

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Cytokine Regulation of Angiogenic Factor Expression by Human Breast Cancer Cells *In vitro*

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Abstract

Background: Angiogenesis is critical for breast cancer tumor growth and metastasis. This process is regulated by chemical signals known as angiogenic factors (AF). One recently identified AF is the cytokine Interleukin 8 (IL-8). ~~Although other angiogenic factors have been implicated in cancer, previously nothing was known regarding the existence and importance of IL-8 in tumor cells in human breast cancer.~~ Recently, we have demonstrated the presence of IL-8 in human breast cancer tissue. ~~This locally produced IL-8 can then support angiogenesis and resulting tumor growth and metastasis.~~ Since in inflammatory diseases, IL-8 expression is known to be under the control of cytokines such as Interleukin 1 (IL-1), and Tumor Necrosis Factor (TNF) we hypothesize, that in the tumor microenvironment, IL-8 production is also under the control of these regulatory cytokines. To test this hypothesis, we evaluated the ability of IL-1 or TNF to regulate the expression of IL-8 in human breast epithelial cells (NBEC) , and human breast cancer cells (BCC).

Methods: Five human BCC, and 3 NBEC were stimulated with IL-1α, IL-1β, TNF-α or TNF-β. The production of IL-8 by the cells was measured by RIA. All data was expressed as IL-8 pg/ml and Stimulation Index (stimulated/unstimulated IL-8 levels).

Results: Generally, baseline (unstimulated) IL-8 expression by NBEC and BCC was extremely low (range 0.1 - 2.3 pg/ml). Analysis of the 24 hour cytokine-stimulated supernatants indicated that there was no stimulation of two BEC s or the estrogen

dependent cell lines with TNF-α or TNF-β. Interestingly, TNF-α and TNF-β induced a 3-24 fold increase in one BEC, and a 2-8 fold stimulation in the estrogen-independent cell lines BCC. When BEC were stimulated with IL-1α or IL-1β, a 5-104 fold stimulation was demonstrated. In the case of the estrogen dependent cell lines, two of the lines (MCF-7 and T-47D) showed little stimulation, while the third (ZR-75-1), showed 120-134 fold IL-8 levels. The two estrogen independent cell lines showed a 330-1138 fold increase in IL-8 expression when stimulated with IL-1 cytokines.

Conclusion: These observations not only demonstrate the ability of human breast cancer cells to produce IL-8 *in vitro*, but indicated that IL-1α and IL-1β are potent inducers of IL-8 expression by BEC and BCC.

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Introduction

Angiogenesis is generally accepted to be critical to tumor growth and metastasis. This process is thought to be controlled by a variety of chemical signals known as angiogenic factors (AF). A number of well established AF such as Vascular Endothelial Growth Factor (VEGF), basic Fibroblast Growth Factor (bFGF), and more recently, Interleukin 8 (IL-8) have been described, and implicated in human cancer. Although the role of IL-8 in other cancers has been reported [von Biberstein, 1995 #155], currently nothing is known about the existence or importance of IL-8 in breast cancer. To begin to fill this gap in our knowledge, we recently investigated IL-8 expression in human breast tissue using immunohistochemical methods [S. Kurtzman, 1994 #401]. In these studies, we found that there was intense IL-8 staining of tumor cells found in samples of human invasive breast cancer specimens. Less intense and inhomogeneous staining was seen in tumor cells in *in situ* breast cancer and only faint, luminal staining of benign breast epithelium in non malignant breast tissue. As a result of our studies, we hypothesized that in the tumor microenvironment, IL-8 is present and associated with breast cancer cells. As a result of these studies, we further hypothesized that BCC can produce IL-8, and that BCC IL-8 expression is directly under the control of IL-8 inducing cytokines, which are likely present in the tumor microenvironment [*i.e.* Interleukin 1 (IL-1) and Tumor Necrosis Factor (TNF)]. In order to directly test this hypothesis, we 1) determined the ability of breast cancer cells (BCC) and breast epithelial cells (BEC) to

produce IL-8 *in vitro,* and 2) determined the ability of IL-1 and TNF to induce IL-8 expression by these cells.

The results of our studies demonstrate not only the ability of human breast cancer cells to produce IL-8 *in vitro*, but further that this expression can be regulated by cytokines such as IL-1 and TNF. Interestingly, IL-1 seems to be an extremely potent inducer of IL-8 expression in estrogen independent breast cell lines. Clearly these *in vitro* studies will provide the foundation for the study of the role of cytokines in the regulation of angiogenesis in human breast cancer, including the use of IL-1 antagonists and inhibitors to suppress IL-8 induced angiogenesis.

Materials and Methods

General reagents

Chicken antibody to IL-8 was prepared by intramuscular injection of 100 μg of recombinant human IL-8 (77 amino acids, Pepro Tech Inc., Rocky Hill, NJ) prepared in *Hunger's* Titer Max (CYTRX Corp., Norcross, GA). Egg yolks containing antibody were processed as previously described (need ref). Antibody titer and specificity were assessed, as previously described (ref., Jenny Qi IL8 VEC, JI). IL-1α and IL-1β, TNFα and TNFβ ELISA were obtained from R & D Corp (Minneapolis, MN).

Cell Culture

All cell lines used for these studies were obtained from ATCC or Clonetics, Corp (Table 1). The cells used in these studies were initially propagated in T-75 flasks, and then plated in 12-well tissue culture plates 24 hours prior to use. Cell viability was assessed in the presence and absence of cytokine using trypan blue exclusion. No cytokine-related cell toxicity was observed. Generally, we determined that 5 X 10^5 to 10^6 cells produced a confluent monolayer of cells when placed in 12-well tissue culture plates. For the purposes of these experiments, the cells used were only tested as confluent cultures.

All cells are grown in recommended media containing 10% fetal calf serum (FCS), and maintained in humidified 5% CO_2 at 37°C. Estradiol was present in FCS at levels of 26 pg./ml. In addition, phenol red is present in all media.

Cytokine stimulation

For cytokine stimulation studies as described above, the NBEC or BCC were allowed to adhere to the plates overnight. Then the individual culture media was aspirated from the plates, and the cells were immediately treated with control media, or media containing the test cytokine (IL-1α, IL-1β, TNFα or TNFβ) at 10 mg/ml. Media used for each cell stimulation was the same media used to keep the cells in continuous culture. Cell cultures were exposed to media or media plus cytokines for 24 hours. At the end of the exposure period, the cell culture supernatants were aspirated, and frozen at -70°C until analyzed. The adherent cells were treated with 0.1% Triton X in PBS until cell lysis was demonstrated microscopically. The resulting cell lysates were then aspirated, and frozen at -70°C until analyzed.

IL-8 RIA

Lauri need to check this paragraph

NBEC and BCC culture supernatants and lysates were analyzed by an IL-8 specific radioimmunoassay (RIA) developed in our laboratory. Briefly, sample (100 µl) was incubated at RT for 1 hour with chicken anti-human IL-8 antibody diluted 1:2,000 in RB buffer (1% BSA in PBS). Human ^{125}I-IL-8 (NEN Products, Boston, MA) diluted in RB buffer (70-80,000 CPM/ml) was added (100 µl) and the reaction mix was incubated for 1 hour at room temperature. Next, 500 µl of fetal bovine serum was added as carrier protein. The immune complex was precipitated out of solution using a 40% saturated ammonium sulfate solution, and pelleted (3000 RPM for 20 minutes), blotted and

counted (gamma counter 1 min.). Samples were quantified by reference to a standard curve constructed using rIL-8 standards (0.039-10 ng/ml). Samples were assayed multiple times and results are expressed as the mean ± S.D.

Standard curves were used to determine the quantity of cytokine in the sample based on the level of radioactivity of each sample using regression analysis. All samples were assayed in duplicate.

Data Analysis

For ease of analysis, the *in vitro* data are expressed as cytokine index (SI). The SI equals the IL-8 level present in the cytokine stimulated cell supernatants divided by the IL-8 levels in the culture supernatants using control media. The data was transformed into natural log to achieve a normal distribution.

The transformed values were then analyzed using the linear regression model from the JMP statistical program. A $p < 0.05$ was evidence of a regression effect and considered statistically significant.

Results

Cell Culture

Both the NBEC and BCC were found to grow well in 12-well tissue culture plates, *i.e.* the cells became adherent and confluent after overnight incubation. Generally, confluent culture wells contained $5x10^5$ - $1x10^6$ cells.

Baseline IL-8 expression

We began our investigation by first determining baseline expression of IL-8 by breast epithelial cells, *i.e.* unstimulated cytokine expression of IL-8 by normal breast epithelial cells (NBEC), virally transformed BEC, estrogen dependent BCC and estrogen independent BCC. The results of the baseline studies are presented in Figure 2 and Table 2 and discussed below. In general, IL-8 levels found in the cell lysates paralleled the levels seen in the supernatants being approximately 10% of the supernatant level. For this reason, the cell lysate data is not presented.

Baseline IL-8 Expression: Breast Epithelial Cells:

Baseline IL-8 expression were initially determined in culture supernatants from normal breast cell lines HMEC, and Hs 578 Bst. As indicated in Table 2, HMEC cells produced 2.3 ± 0.43 pg./ml IL-8 as compared to 0.48 ± 0.07 pg./ml for the Hs 578 Bst. Parallel analysis of the cell lysates demonstrated low levels of cell associated IL-8 in both normal breast cell cultures (*i.e.* <1 pg./ml). Interestingly, the SV-40 transformed BEC cell

line, HBL-100 demonstrated significantly higher baseline IL-8 levels (*i.e.* 8.8 ± 1.43 pg./ml) in the cell culture supernatants when compared to either of the normal BEC lines (HMEC 3.83 fold, Hs 578 Bst 18.33 fold). This elevation of the IL-8 levels in the HBL-100 cells was also seen in the cell associated lysates (4.7 pg./ml). This elevation of the IL-8 levels in the HBL-100 cells was not seen in the cell associated lysates (<1.0 pg./ml).

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Baseline IL-8 Expression: Estrogen Dependent Breast Cancer Cell Lines

After establishing baseline expression of IL-8 in the non malignant breast cell lines, we next examined the baseline levels in three human estrogen dependent breast cancer cell lines, MCF-7, T-47 D, and ZR-75-1 (see Table 2). All three cell lines showed extremely low levels of baseline IL-8 expression in the cell culture supernatants (range: 0.1-0.3 pg./ml) and lysates (range: 0.07-0.15 pg./ml). These levels were 10-50% of those seen in the normal BEC.

Baseline IL-8 Expression: Estrogen Independent Breast Cancer Cell Lines

Baseline IL-8 levels in cell culture supernatants and lysates were determined in the two estrogen independent human breast cancer cell lines, BT-20 and MDA-MB-231. Much like the estrogen dependent BCC, the BT-20 and MDA-MB-231 cell culture supernatants showed low levels of IL-8 expression in unstimulated conditions (*i.e.* <1.0 ng/ml). The levels in the cell lysates were similarly low (<1.0 ng/ml).

TNF stimulation of IL-8 Expression

In order to test whether TNF-α or TNF-β could induce IL-8 expression from breast cells, we measured the levels of IL-8 in 24-hour cell culture supernatants and lysates from NBEC and BCC treated with either TNFα or TNFβ (see Figure 1 and Table 2). The results of these studies are presented below. Our data indicate that there is a heterogeneous response of breast cells to TNF stimulation. This phenomenon likely reflects the pattern seen in normal breast tissue and in patients with malignancies. (Mention receptors in the Discussion section)

TNF Stimulation: Normal Breast Epithelial Cells

In an effort to establish the effects of TNFα and TNFβ on IL-8 expression in normal breast epithelial cells, we utilized the cell lines HMEC and Hs 578 Bst. In the case of Hs 578 Bst, TNFα induced a 23.60-fold stimulation of IL-8 expression when compared to unstimulated (media) control supernatants [*i.e.* stimulation index (SI)=23.60]. Interestingly, there was minimal stimulation of IL-8 by TNFα with the HMEC cells (SI=1.31). A similar pattern of IL-8 stimulation was seen for TNFβ (see Figure 1 and Table 2). Thus, TNFα and TNFβ stimulated the Hs 578 Bst BEC, but not the HMEC. Finally, the transformed BEC HBL-100 showed only modest stimulation of IL-8 expression by TNFα (SI=2.05) or TNFβ (SI=1.35).

TNF Stimulation: Estrogen Dependent Breast Cancer Cell Lines

We next tested the ability of TNF to induce IL-8 expression in the estrogen dependent BCC. As can be seen in Table 2, neither TNFα or TNFβ stimulation resulted in induction of IL-8 by any of the estrogen dependent cell lines. In fact in the case of the ZR-75-1 BCC there was modest inhibition of IL-8 expression. This inhibition was not a result of cell toxicity or cell loss as judged by trypan blue exclusion and cell counting. Check this with Lauri/literature. Thus neither TNFα or TNFβ appear to be inducers of IL-8 expression by estrogen dependent BCC.

TNF Stimulation: Estrogen Independent Breast Cancer Cell Lines

In parallel studies, we examined the ability of TNFα and TNFβ to induce IL-8 expression in estrogen independent BCC. We found that there was modest induction of IL-8 in the MDA-MB-231 BCC by TNFα (SI=6.78) and TNF-β (SI=1.89). In contrast, there was no IL-8 stimulation seen in the BT-20 cells in response to either TNF-α or TNF-β.

IL-1 stimulation of IL-8 Expression

IL-1 cytokines are known to be a potent inducers of IL-8 expression in leukocytes and normal tissue cells [Cohen, 1995 #137]. Therefore, we extended our studies to investigate the ability of IL-1α and IL-1β to induce IL-8 expression in normal and malignant breast epithelial cells.

IL-1 Stimulation: Breast Epithelial Cells

Both normal BEC lines showed marked increased expression of IL-8 when treated with either IL-1α or IL-1β (Table 2). There was approximately a 10-fold difference in the responsiveness of the two cell lines (HMEC SI=10.91, Hs 578 Bst SI=80.03) in response to IL-1 α, as well as in response to IL-1 beta (HMEC SI=6.56, Hs 578 Bst SI=103.81). This observation emphasizes the heterogeneous responsiveness between cell lines, as well as between various cytokines.

Analysis of the of induction of IL-8 expression in SV-40 transformed BEC line HBL-100 in response to IL-1α or IL-1β IL-1 stimulation demonstrated that both IL-1α and IL-1β induced high levels of IL-8 expression (IL-1α SI=6.88, IL-1β SI=5.24) in these cells. The only cell line with significant amounts of IL-8 in the lysates was the IL-1α and IL-1β stimulated MDA MB 281 cells, in this line the amount in the lysate was 25-33% that found in the cell culture supernatants. This data demonstrates that IL-1 is a potent inducer of IL-8 in non malignant BEC.

IL-1 Stimulation: Estrogen Dependent Breast Cancer Cell Lines

Evaluation of the ability of IL-1α or IL-1β to induce IL-8 expression in estrogen-dependent cell lines demonstrated a clear heterogeneity in IL-8 response (see Table 2). For example, MCF- 7 cells showed no response to stimulation (IL-1α SI=1.08, IL-1β SI=1.37). The T-47 D BCC showed intermediate responses to IL-1 stimulation (IL-1α

SI=2.89, IL-1β SI=3.23), and the ZR-75-1 BCC demonstrated profound stimulation (IL-1α SI=120.26, IL-1β SI=133.99). These data demonstrate once again the heterogeneity in IL-8 expression in estrogen dependent BCC lines. Further, this data clearly shows that these cells can be induced to produce IL-8 and that IL-8 rather than TNF in these cells consistently stimulated IL-8 expression.

IL-1 Stimulation: Estrogen Independent Breast Cancer Cell Lines

In order to evaluate the ability of IL-1 cytokines to induce IL-8 expression in estrogen independent BCC, we measured the IL-8 expression in cell culture supernatants and lysates at 24 hours in response to IL-1α or IL-1β stimulation. As can be seen in Tables 2 and 3, IL-1 cytokines were extremely potent inducers of IL-8 expression in both cell lines. IL-1α and IL-1β induced SI of over 300-fold in the MDA-MB-231 cells, and approximately 1,000-fold in the BT-20 BCC. This data not only emphasizes the differential expression of IL-8 by benign and malignant breast cells in response to various cytokines *in vitro*, but also demonstrates that a marked heterogeneity exists in breast cell responsiveness *in vitro*. *In vivo*, this heterogeneity in tumor response to IL-1 and TNF suggests that heterogeneity of AF expression (*e.g* IL-8 vs. VEGF) likely controls tumor growth and metastasis.

Discussion

It is estimated that there will be 180,000 new cases of breast cancer diagnosed in the United States in the next year. Seventy thousand (70,000) women are expected to die of their disease. While surgery has been the primary therapeutic modality employed for these patients, despite adequate surgery, a substantial number of women will die of metastatic disease. Adjuvant chemotherapy has been effective in increasing survival by approximately 15% for any given stage of disease. Chemotherapy will fail in the remainder of the patients. Many factors beyond tumor size, and lymph node involvement have been examined to try and identify those patients who are likely to have micro-metastatic disease at the time of prior resection. Recently, angiogenesis has been utilized as a prognostic factor in breast cancer patients [Heimann, 1996 #459]. It is a generally accepted principle that tumors cannot grow beyond several millimeters in diameter without recruiting new blood vessels (*i.e.* angiogenesis). -OR- Recently tumor angiogenesis has been proposed as a prognostic factor in predicting cancer patient outcome [Heimann, 1996 #459]. A considerable body of evidence has demonstrated that angiogenesis in invasive breast cancer is associated with poor outcome as measured by shorter disease-free survival, and increased frequency of metastatic disease.

Angiogenesis and Breast Cancer

Angiogenesis, or neovascularization, has long been known to be a central aspect of tumor growth and metastasis. Angiogenesis is not only critical in allowing the influx of

gases and nutrients into the rapidly proliferating tumor micro-environment (TME), but also provides a route for dissemination of the tumor cells to distant sites within the body. Recently, it has become clear that patients whose tumors demonstrate a high degree of angiogenesis have a shorter disease-free survival, and that these patients are more likely to develop distant metastases [Weidner, 1991 #34]. This phenomenon has now been described in a number of human cancers including breast [Weidner, 1992 #28], lung [Yamazaki, 1994 #327; Fontanini, 1995 #338; Jaeger, 1995 #339; Macchiarini, 1994 #323], prostate [Weidner, 1993 #320; Fregene, 1993 #324], rectal [Saclarides, 1994 #333], testicular [Olivarez, 1994 #331], bladder [Jaeger, 1995 #339], melanoma [Cockerell, 1994 #345; Barnhill, 1993 #321; Guffey, 1995 #336; Barnhill, 1992 #340], and myeloma [Vacca, 1994 #325; Vacca, 1995 #319]. In a review of 211 node negative breast cancer, the number of intratumoral vessels was confirmed to be a highly significant factor in relapse free survival and overall survival [Bevilacqua, 1995 #388]. In a study by Fox *et al*, vascular counts in primary tumors predicted both relapse free survival (RFS) and overall survival (OS) in breast cancer specimens, while in that review, ER receptor status did not [Fox, 1994 #6]. The presence of endothelial growth factor receptor (EGFR) also was associated with a significant reduction in RFS in highly vascularized tumors in that study. A positive relationship between VEGF, a potent angiogenic factor, and microvessel density was demonstrated by Toi *et al* [Toi, 1994 #22; Toi, 1995 #389]. In those studies, a significant relationship was found between microvessel density and RFS in both node negative, and node positive breast cancer patients. The number of microvessels correlated positively with the number of histologically positive nodes. Thus

angiogenesis has been shown to be an important prognostic feature in invasive breast cancer.

The development of angiogenesis is under the regulation of molecules called angiogenic factors (AF) [Folkman, 1987 #44]. Interestingly, while the identity of these AF has been described, and the importance of the process well accepted, little is known about the regulation of AF expression by human cancers. Understanding the underlying mechanisms and factors that control angiogenesis will provide not only insights into the basic mechanisms of tumor growth and metastasis, but also will likely provide novel new therapeutic approaches to invasive breast cancer.

AF are known to control angiogenesis by inducing vascular endothelial cell (VEC) proliferation and migration. Major established AF include IL-8, VEGF, bFGF and TGFβ. In addition to these AF, angiogenesis is also regulated by AF receptors (AFR) on VEC. In the case of IL-8, two receptors designated IL-8 receptor A and receptor B have been identified. VEGF also has had two receptors identified designated Flt and Flk. FGFβ has had four distinct receptors characterized which are designated FGFR1-FGFR4. Finally, two receptors for TGFβ have been identified and designated TGFβ R1 and TGFβ R2. Clearly, the interplay of AF, AFR and AF antagonists determine not only the occurrence of angiogenesis but the extent to which it occurs. Clearly, the interplay of angiogenic factors, receptors and antagonists determine not only the occurrence of angiogenesis but the extent to which it occurs.

Cytokines Regulation of AF Expression

Cytokines have been implicated as important regulators in wound healing, as well as in inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, and interstitial lung disease.14 It has been postulated that in instances of chronic inflammation and in autoimmune diseases, proinflammatory cytokines are present in the tissue microenvironment in quantities sufficient to perpetuate the inflammatory process. Additionally, altered production and response to cytokines have often been described in malignancy.[9] Conceptually, tumors are often viewed as "wounds that will not heal."[15] We have extended this concept to dissect the role of cytokines such as IL-1, TNF, and IL-8 in cancer. Recently, we have demonstrated elevated IL-8 levels associated within HNSCC tumor cells.[6] IL-8 is a member of a family of 8-10 kDa cytokines that are involved in proinflammatory and reparative processes.[4,5] Cytokines in this family are basic heparin binding proteins that display chemotactic activities for vascular endothelial cells (VEC) *in vitro* and neovascularization *in vivo*.[16] Recently, it has been shown that IL-8 is also a growth factor for human melanoma cells[17] and that it mediates the neovascularization and inflammation in bronchogenic carcinoma tissue.[18] Additionally, we have demonstrated that *in situ* IL-8 is a prominent immunohistologic feature of human breast cancer cells.[19] These data, together with our previous observations of IL-8 production by head and neck cancer cells[6], suggests that IL-8 may be an important marker for the invasive properties of human tumors and likely contributes to angiogenesis. These studies not only underscore the importance of IL-8 in cancer, but raise the question of its regulation by factors such as IL-1 and TNF.

IL-1 and Cancer

IL-1 is a term for two polypeptides, IL-1α and IL-1β, that possess a wide spectrum of inflammatory, metabolic, physiologic, hematopoietic, and immunologic properties.[20] Although the term interleukin means "between leukocytes," IL-1 is synthesized by many different cells, including leukocytes; macrophages; astrocytes; endothelial cells; smooth muscle cells; fibroblasts; synovial lining cells; dermal dendritic cells; keratinocytes; intestinal, gingival, and cervical epithelium; natural killer cells; and maternal placental cells.[20] Because IL-1 is rapidly synthesized by nearly every nucleated cell when stimulated, is present in many different organs, and is associated with numerous biological effects, it is considered a "prime controller" cytokine in the hierarchy of pro-inflammatory and pro-tumorigenic cytokines. IL-1α and IL-1β bind to two IL-1 receptor molecules, an 80-Kd receptor (IL-1RtI), and a 68-Kd receptor (IL-1RtII).[20] In general, IL-1a binds preferentially to the type I receptor, and IL-1β binds optimally to the type II receptor.[20] The biological activity of IL-1 is regulated at several levels, including gene expression, processing and release, receptor binding, and antagonists, including IL-1RA.[12]

TNF and Cancer

TNFα and tumor necrosis factor beta are known to have both pro-tumorigenic and anti-tumorigenic activities *in vitro* and *in vivo*. These beneficial and detrimental effects of tumor necrosis factors on tumor growth and metastases remain controversial [6]. Tumor

necrosis factor alpha was initially functionally described in 1893 by Dr. William Coley who noted hemorrhagic necrosis of a sarcoma in a patient with a concomitant bacterial infection [7]. In 1962, O'Malley *et al* reported that the injection of serum obtained from animals treated with bacterial lipopolysaccharide into another tumor-bearing animal induced the hemorrhagic necrosis of the tumor [7]. Twenty years later, Lloyd Old *et al* discovered that the capacity to induce the necrosis of certain mouse tumors *in vivo* and *in vitro* could be ascribed to a factor present in the blood after lipopolysaccharide injection [8]. At the same time, Anthony Cerami discovered that cachexia associated with hypertriglyceridemia in rabbits chronically infected with *T. brucei* resulted from the presence of a serum protein which he call "cachectin" [8]. This protein was later termed tumor necrosis factor and was subsequently designated tumor necrosis factor alpha when a related cytokine with similar actions was identified, termed tumor necrosis factor beta (TNFβ), or lymphotoxin [8].

TNF has an anti-proliferative effect against several transformed neoplastic cell lines *in vitro* [5]. The exact mechanism of the anti-tumor action of TNF is unknown. Direct anti-tumoral effects *in vitro* are observed for TNF alone, and cytotoxicity of tumor necrosis factor is potentiated by other agents (cytokines, chemotherapeutics, radiation, and hyperthermia) [5]. Toxicity against tumor cells may be induced by TNF itself, depending upon the specific tumor cell sensitivity to TNF or through other mechanisms such as release of superoxides by macrophages, cytotoxic lymphocytes, or natural killer cells [8]. In mouse tumor models the intravenous injection of TNF induces a hemorrhagic necrosis which is prevented by anti-TNF antibody injection [8]. It is felt that the hemorrhagic

necrosis is the consequence of endothelial cell damage. These findings suggest that, in addition to its toxic effects for malignant cells, TNF likely exerts indirect anti-tumor activity by stimulating the lytic function of the host inflammatory cells.

Few studies have investigated the interactions of TNF and its receptors in head and neck squamous cell carcinoma. Gallo *et al* noted increased tumor necrosis factor production by activated monocytes from patients with head and neck squamous cell carcinoma, but there was no relationship to cancer stage or cancer-related weight loss [9]. Another *in vitro* investigation revealed that head and neck squamous cell carcinoma cell lines exposed to TNFα were resistant to cell death and continued to thrive. Clinical trials in head and neck squamous cell carcinoma have demonstrated that chemotherapy using TNFα alone resulted in no anti-tumor effects, but TNFα combined with interferon gamma or with dactinomycin and doxorubicin led to a synergistic effect which inhibited tumor growth and actually led to tumor regression [10,11].

Although TNF has been implicated in hemorrhagic necrosis seen in certain tumors, TNF has also been noted to facilitate tumor growth and may function as an autocrine growth factor for tumor cells and fibroblasts [12]. TNF activates enzyme cascades that may enhance tumor spread (*e.g.*, metalloproteases), stimulates angiogenesis *in vivo*, and increases the adherence of tumor cells to endothelium *in vitro* [6]. It has also been shown that TNF promotes the invasive growth of tumor cells in the peritoneum and their establishment as tumor nodules below the mesothelial surface [6]. These studies suggest that TNF not only stimulates tumor growth, but it also appears to play an active role in

tumor metastasis. To fully understand the role of TNF in head and neck squamous cell carcinoma, it is important to investigate those factors which control its expression.

Restate hypothesis general

Note c Thus, angiogenesis is an important process that occurs in human breast cancer, IL-8 is an established angiogenic factor, and IL-8 is found in breast cancer specimens demonstrated by immunohistochemical methods. Experimental evidence has shown that blocking angiogenic factors will inhibit angiogenesis in *in vivo* models [Hu, 1994 #53]. Understanding the mechanisms that control this process will likely lead to new treatment strategies to prevent the growth and spread of breast tumors.

These observations led us to hypothesize that in the tumor microenvironment, breast tumors themselves produce AF. These angiogenic factors then act in an autocrine and paracrine fashion and support tumor growth and metastasis. Further we hypothesize that this production of AF is under the control of cytokines such as IL-1 and TNF.

IL-1 and TNF Induction of IL-8 Expression in Breast Cancer

note g Our data clearly demonstrates that human breast cells are capable of producing angiogenic factors such as IL-8. We found that breast cells grown in culture produce low levels of IL-8. Interestingly, the virally transformed non malignant cell line expressed the highest levels of IL-8 expression in the unstimulated state. More importantly, we found that cytokines are potent stimulants for IL-8 production. Specifically, IL-1 is the most potent inducer of IL-8 production. Further, this induction may be related to the estrogen dependence of the breast cells. Both of the estrogen independent breast cancer cells

tested were highly sensitive to IL-1 stimulation, where 2 of the 3 estrogen dependent breast cancer cell lines did not increase production of IL-8 in response to cytokine stimulation.

This data supports our general hypothesis that in the tumor microenvironment, breast cancer cells regulate their own growth through the production of angiogenic factors. In our earlier studies we demonstrated by immunohistochemical analysis that IL-8 antigen was present in breast cancer tissue from patients with breast cancer. The present study demonstrates that the breast cancer cells themselves are the likely source of the IL-8 seen in the tissues. Further, the production of this IL-8 is likely under the influence of cytokines such as IL-1 and TNF.

———This observation is consistent with our approach that cancers behave like wounds that continue to heal in an abnormal fashion. IL-1 and TNF are normally produced by leukocytes which can infiltrate wounds and tumors. In this paradigm, the intercellular signals designed to promote wound healing and repair are contributing to the continued growth of the tumors. This occurs because of the abnormal and uncontrolled response of the cancer cells to the signals, i.e. the production of angiogenic factors.

Significance: General Model (wounds like cancers)

———alternate paragraph 1 The transformation from a mature, differentiated epithelial cell to a malignant cell, capable of growth and metastasis is a multi-step process. In order

to assume a malignant phenotype, the cells must develop the ability to be nourished, invade into the lymphatic or vasculature system, and establish growth at distant sites.

Early on in this process, the cancers behave much like healing wounds (Table 2). Like wounds, the tumors recruit leukocytes, promote fibrin deposition, and establish a blood supply. In the healing wound, the signals for these activities are eventually turned off, and the healing process ends. We and others view cancers as persistent sources of irritation, that perpetuate this healing process, in an abnormal, uncontrolled fashion [Whalen, 1990 #91; Dvorak, 1986 #94]. Further, it is our belief that the tumor cells facilitate this in a paracrine fashion, by overreacting to normal healing signals with the production of, among other things, angiogenic factors such as IL-8. Those tumors that are successful in this process, go on to become clinically apparent cancers, and produce metastases. We expect, that there are other cells undergo malignant degeneration, but be incapable of production of those factors needed to sustain their growth. These tumors would be judged clinically benign, or might not grow to a size where they could be detected. Their fate then would be to remain unnoticed, or to eventually detected by immune surveillance and be destroyed.

Alternate paragraph It has been stated that cancers behave like wounds that won't heal [Whalen, 1990 #91; Dvorak, 1986 #94]. Tumors and wounds share many features such as infiltration with leukocytes, fibrin production, and angiogenesis. We feel that at the tissue level, tumors produce a chronic irritation. This irritation leads to an attempt to heal the area. In order to accomplish this, infiltrating leukocytes enter the area, and release cytokines such as Interleukin 1 (IL-1). These mediators then begin the healing process by

recruiting the cells needed, inducing inflammation, and neovascularization. In healing wounds, this process is self limited. In cancers however, these processes may be over-expressed, and result in support for tumor growth and metastasis. We hypothesize that those malignant cells that are capable of responding to these stimuli by producing angiogenic factors (AF) then go on to form tumors that are clinically apparent. The resulting neovascularization also allow access of the cancer cells to the vascular and lymphatic system and can thus facilitate metastasis. Those cancerous cells that do not respond can not grow, and remain indolent.

Conclusions

In this study, we have demonstrated that breast cancer cells are capable of producing angiogenic factors. In addition, the production of angiogenic actors by breast cancer cells is regulated by cytokines. This data supports our observation that cytokines such as IL-1, TNF and IL-8 found in breast cancer tissue, and supports our hypothesis that in the tumor microenvironment, breast cancer cells support their own growth in an autocrine fashion by producing angiogenic factors and perhaps other growth factors. We anticipate that in the future, agents that block angiogenic factors will be useful in the treatment of breast cancer patients.

Abstract Form

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EXPRESSION OF TUMOR NECROSIS FACTOR (TNF-α AND TNF-β) AND TNF RECEPTORS IN HUMAN BREAST CARCINOMA.

Javid Moslehi, Scott H. Kurtzman*, Yanping Wang, Kathleen H. Anderson, Lauri J. Miller, and Donald L. Kreutzer, Department of Surgery, University of Connecticut School of Medicine, Farmington, CT

INTRODUCTION: Tumor Necrosis Factor (TNF-α and TNF-β) are pleiotropic cytokines whose various biological activities are mediated through two distinct receptors, TNF receptor 1 (TNFR1/p55) and TNF receptor 2 (TNFR2/p75). Previously, TNF was thought to possess only antitumor activities. Recently, a growing body of literature suggests that TNF may also have protumorigenic activities. This led us to hypothesize that *in vivo* tumor cells can express both TNF, as well as TNF receptors. We speculate that the localized expression of TNF and TNF receptors likely plays a key role in tumor cell activation (e.g. proliferation) in the tumor microenvironment.

Type Abstract Within Borders

METHODS: To determine if TNF or its receptors are expressed in breast tumor cells, commercial antibodies and immunohistochemical techniques were used to assess the distribution of TNF-α, TNF-β, TNFRI and TNFRII in 30 malignant (20 invasive, 10 ductal *in situ* (DCIS)) and 10 benign (i.e. fibrocystic disease) breast tissue specimens.

RESULTS: Tumor cell TNF-α expression was detected in 19 out of 20 breast cancer specimen and 6 out of 10 DCIS specimens. TNF-α was also detected on ductal epithelial cells of and 5 out of 10 benign specimen. TNF-β results were similar. Both TNF receptors were present in all tumor cells, in invasive (20/20), as well as DCIS (10/10). TNF expression was significantly decreased in duct cells in benign disease.

CONCLUSION: Our demonstration of enhanced tumor cell expression of TNF and TNF receptors, when compared to normal ductal epithelial cells, suggests both autocrine and paracrine regulation of tumor cells occurs in malignant breast disease. Future therapeutic strategies targeting both TNF and TNF receptors on cancer cells within the tumor microenvironment will likely provide a new approach to controlling tumor growth and metastasis in human breast cancer.

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EXPRESSION OF INTERLEUKIN 1 RECEPTORS IN HUMAN BREAST CANCER

Interleukin 1 (IL-1) is a pleuripotent cytokine that has been demonstrated to be a key regulatory agent in cell activation and growth. Recent work in our laboratory has demonstrated the presence of IL-1 cytokines (i.e. IL-1α and IL-1β) in human breast tissue homogenates and human breast cancer cells. We have also demonstrated the ability of IL-1 to regulate the expression of the pro-angiogenic cytokine Interleukin 8 in an *in vitro* model of human breast cancer cells. Clearly, in order for IL-1 to exert it's effect, there must be receptors for IL-1 present on the cells. Currently there are two known IL-1 receptors designated as IL-1 rI, and IL-1 rII. Only IL-1 rI is known to transduce IL-1 signals within the cells. IL-1 rII is non signal transducing and is thought to act as a trap for excess IL-1. The present study was undertaken to characterize the presence and distribution of IL-1 receptors in human breast specimens.

In our present studies, we evaluated breast specimens from 30 patients with invasive breast cancer (IBC), 8 patients with ductal carcinoma in situ (DCIS) and 8 patients with non atypical proliferative breast diseases (NB). Immunohistochemical analysis was carried out for both IL-1 rI and IL-1 rII using receptor specific antibodies and standard ABC immunohistochemical techniques. In the case of IL-1 rI, a specific blocking peptide was used as a control for antibody staining specificity.

Our studies demonstrated that in essentially all (>98%) specimens from patients with IBC and DCIS, tumor cells stained positively for IL-1 rI and IL-1 rII. We also detected IL-1 rI and IL-1 rII in ductal epithelial cells in IBC, DCIS and NB. IL-1 rI and IL-1 rII were expressed on vascular endothelial cells of both large and small blood vessels found in these specimens. Interestingly, while IL-1 rI was found in fibroblasts in approximately 50% of the specimens in each patient group, IL-1 rII was only found in fibroblasts from 50% of the specimens of patients with IBC, but in none of the specimens from patients with DCIS or NB.

These observations support our general hypothesis that IL-1 is an important regulatory cytokine in human breast cancer. In the future, these receptors may be targets for therapeutic intervention with receptor targeted antibodies or antagonists.

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EXPRESSION OF INTERLEUKIN-8 RECEPTORS ON TUMOR CELLS AND VASCULAR ENDOTHELIAL CELLS IN HUMAN BREAST CANCER TISSUE : Implications for Tumor Proliferation and Angiogenesis

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ABSTRACT

A growing body of literature has implicated cytokines as key elements in tumor growth and metastasis. Recently, we confirmed the presence of the cytokine Interleukin-8 (IL-8) in tissue from patients with breast cancer. For this study, we hypothesize that the IL-8 receptors are also present and that these receptors play an important role in controlling tumor cell activation (e.g. proliferation) and vascular endothelial cell (VEC) activation (e.g. angiogenesis) in human breast cancer. Immunohistochemical analysis for the IL-8 receptors (IL-8RA & IL-8RB) was performed on 43 malignant and 8 benign breast tissue samples. In the malignant samples, the tumor cells expressed both IL-8RA & IL-8RB in all of the specimens tested. The small vessel endothelial cells (SVEC) expressed IL-8RA in 65% and IL-8RB in 95% of the samples. Interestingly, limited large vessel endothelial cell (LVEC) staining was evident for IL-8RA (14%) but was prominent for IL-8RB (70%). In the benign breast samples, IL-8RA was expressed by the ductal epithelial cells (DEC) in 50%, while IL-8RB was noted in 37% of the specimens. The SVEC showed consistent expression of both receptors (88%). While limited IL-8RA expression (13%) was noted for the LVEC, the expression of IL-8RB was present in 75% of the samples. Our results clearly demonstrate that both tumor cells and VEC express the IL-8 receptors. We believe that the expression of IL-8 and its receptors is key to regulating tumor and VEC activation which controls proliferation, angiogenesis and metastasis in human breast cancer.

INTRODUCTION

Tumor growth and metastasis are dependent on a variety of processes including tumor invasion, angiogenesis, tumor migration and tumor proliferation to name but a few. These processes are generally believed to be under the control of a variety of chemical signals such as angiogenic factors (AF), migration factors, and growth factors. Central to this system of chemical signals are small molecular weight glycoproteins known as cytokines and their associated receptors.

Recently the cytokine Interleukin 8 (IL-8) has been shown to be both a potent AF and a growth factor in both normal and disease states. For example, IL-8 has been proven to stimulate angiogenesis in the normally avascular environment of the rat and rabbit cornea[1,2] as well as to promote proliferation in a variety of normal cell types including human glioma cells[3], keratinocytes[4], and vascular smooth muscle cells[5]. Additionally, IL-8 has been shown to be present in many neoplastic diseases including in cultured melanoma cells[6] and in prostate[7], head and neck[8], brochogenic[9] and colorectal[10] cancers.

Previously we have reported IL-8 expression on tumor cells and on the VEC in human breast cancer tissue. (Kurtzman *et.al.*, Abstract presented at Society of Surgical Oncology, March 1994) For the functional expression of IL-8 to occur, both IL-8 and the IL-8 receptors must be present. Currently, nothing is known regarding the expression of the IL-8 receptors (IL-8RA and IL-8RB) in breast cancer or in any other neoplastic disease. For the present study, we hypothesized that IL-8 receptors are present on the tumor as well as on the VEC (within the tumor microenvironment) in human breast cancer. To test this hypothesis, we determined the distribution of the two IL-8 receptors (IL-8RA, IL-8RB) on

cells in tissue from malignant and benign human breast disease patients using immunohistochemical techniques. Our studies clearly demonstrate that IL-8RA and IL-8RB are present on both tumor cells and VEC, and support our hypothesis on the role of the IL-8 system in human breast cancer.

We believe, that IL-8 and IL-8 receptor expression play a dual role, i.e. they are important in tumor cell proliferation as well as in angiogenesis. We speculate that the breast cancer tumor cells secrete IL-8 which when recognized by the IL-8 receptor, leads to autocrine-paracrine proliferation of the tumor. In addition, IL-8 receptor presence on the VEC, allows the endothelial cells to recognize IL-8 and respond to it by forming new blood vessels. These new vessels supply the tumor with the nutrients it needs for growth as well as provide a means for tumor metastasis. In the future, agents that interfere with the IL-8 system may be useful in the treatment of patients with breast cancer.

RESULTS

Our initial effort was focused on determining the presence and distribution of IL-8RA and IL-8RB on tumor cells and VEC in tissue from breast cancer as well as benign breast disease patients. The results of these studies are presented below and summarized in Table 1. For all of the immunohistochemical analysis performed in this study, a set of controls was run to verify the specificity of the antibody reaction. All of the control slides were negative, and showed no evidence of staining. Figure 1 (C,F,I,L) shows an example of these control slides.

MALIGNANT HBC SPECIMENS - TUMOR CELL EXPRESSION

For IL-8 to be functional as a cell activator, (e.g. a growth factor, AF, etc.) it would be essential for tumor cells to express a receptor to recognize IL-8's signal. For this reason we set out to explore whether the IL-8 receptors are present on HBC tumor cells.

Malignant tumor cell IL-8RA expression : All of the invasive (36/36) and DCIS (7/7) HBC specimens stained positively for the IL-8RA receptor on tumor cells. Interestingly, IL-8RA expression showed a wide degree of variation of staining within a tumor, i.e. staining tended to be heterogeneous, associated with single cells or an area of tumor cells. In well differentiated tumors, with abortive duct formation, staining was in an apical, circular pattern. A number of malignant specimens examined contained areas of normal or hyperplastic ductal morphology adjacent to the tumor. The majority of these areas expressed the IL-8RA on the ductal epithelial cells. Again, as seen in the malignant samples, staining was in an apical pattern, with intense staining closest to the lumen of the duct. The myoepithelial cells surrounding the ductal epithelial cells showed little evidence of IL-8RA expression.

Malignant tumor cell IL-8RB expression : All invasive (36/36) and DCIS (7/7) HBC specimens stained positively for the IL-8RB receptor on tumor cells. Unlike the heterogeneous, cell associated staining displayed for IL-8RA, the staining pattern for IL-8RB was homogeneous throughout the tumor with only slight variations within a specimen. On samples where areas of normal or hyperplastic ductal morphology was present adjacent to the tumor, less than half of the samples showed IL-8RB expression on the ductal epithelial cells. When staining in the non-neoplastic specimens was present, it was not in the apical pattern apparent for IL-8RA expression. Almost all of the myoepithelial cells expressed the IL-8RB. In summary, all of the HBC specimens examined showed that tumor cells express both the IL-8RA and IL-8RB. These results demonstrate that if IL-8 is indeed acting as a growth factor, it can be recognized by the tumor cells in HBC. Figure 1(A-B) displays the tumor cell expression of IL-8RA and IL-8RB.

MALIGNANT HBC SPECIMENS : VEC EXPRESSION

For IL-8 to act as an AF, it would be necessary for the VEC to express IL-8 receptors to recognize IL-8's signal to undertake the angiogenic process. For this purpose, we explored whether IL-8RA and IL-8RB were expressed on the SVEC as well as the LVEC.

Malignant SVEC IL-8RA expression : In the invasive HBC, 67% (24/36) and in DCIS, 57% (4/7) of the samples showed some positive staining on the SVEC for IL-8RA. Overall, or 65% (28/43) of the malignant HBC patients showed IL-8RA expression. Even though positive SVEC's were located in 28 of the slides, only 11 (25%) had SVEC staining that was clearly evident while the remainder showed areas of positive and negative SVEC staining.

Malignant SVEC IL-8RB expression : A far different picture was present for IL-8RB expression on SVEC. In the invasive samples, 94% (34/36) and 100% (7/7) of DCIS, were positive for IL-8RB expression. A total of 95% (41/43) of the malignant specimens showed positive staining for this receptor. The SVEC's were very visible and easy to locate in the majority of slides.

Malignant LVEC IL-8RA expression : In the invasive samples only 17% (6/36) while none of the DCIS specimens showed expression of IL-8RA on the LVEC. Overall, 14% (6/43) of the malignant samples showed positive staining for IL-8RA. Figure 1J shows the typical negative staining of the LVEC, while the smooth muscle surrounding the arterial vessels consistently expressed IL-8RA.

Malignant LVEC IL-8RB expression : The expression of IL-8RB on the LVEC was distinctly different from that of IL-8RA. 72% (26/36) of the invasive and 57 % (4/7) of the DCIS, for a total of 70% (30/43) of the malignant samples showed positive staining for IL-8RB. The smooth muscle surrounding the arterial large vessels stained intensely for IL-8RB in the majority of the malignant specimens studied. From our investigation of the expression of IL-8RA and IL-8RB by the VEC, it is evident that while both IL-8RA and IL-8RB can be expressed, IL-8RB is the prominent receptor present on the SVEC and LVEC. An example of SVEC and LVEC expression of IL-8RA and IL-8RB can be seen in figure 1 (G,H,J,K)

BENIGN BREAST DISEASE SPECIMENS-

One of our objectives in testing benign breast disease samples was to discover whether a difference existed between the expression of the IL-8

receptors in these samples when compared to the malignant specimens. We examined 8 fibrocystic breast tissues for the expression of IL-8RA and IL-8RB on the ductal epithelial cells (DEC), SVEC and LVEC.

BENIGN BREAST DISEASE SPECIMENS : DEC EXPRESSION

Benign DEC IL-8RA expression : Expression of IL-8RA on the ductal epithelial cells occurred in 50% (4/8) of the specimens. All of these displayed staining in an apical pattern, on the portion of the cell closest to the lumen of the duct. 88% (7/8) showed IL-8RA expression on the myoepithelial cells surrounding the ductal epithelium.

Benign DEC IL-8RB expression : Only 37% (3/8) of the samples tested stained for IL-8RB on the ductal epithelium. Two of the three showed the apical pattern of staining evident with IL-8RA expression. All (8/8) of the specimens showed expression of IL-8RB on the myoepithelial cells.

In summary, only about 50% of the benign DEC samples expressed IL-8RA or IL-8RB, where as the majority of the myoepithelial cells surrounding the ducts expressed these receptors. Expression of IL-8RA and IL-8RB by the ductal epithelial cells is displayed in figure 1(D-E).

BENIGN BREAST DISEASE SPECIMENS : VEC EXPRESSION

Benign SVEC IL-8RA & IL-8RB expression : The majority of the benign breast disease samples (88%) showed positive staining for both IL-8RA and IL-8RB. As in the malignant samples, most of the samples showed positive and negative areas of SVEC IL-8RA expression, while IL-8RB expression by SVEC's was clearly present through out the specimens.

Benign LVEC IL-8RA & IL-8RB expression : Only 13% (1/8) of the samples tested were positive for IL-8RA on the LVEC in the benign breast

samples whereas the majority, 75 % (6/8), were positive for IL-8RB. The smooth muscle surrounding the LVEC stained positively in 63% (5/8) of the samples tested for both of the receptors. In summary, the majority of SVEC's expressed both IL-8RA and IL-8RB. As was seen in the malignant samples, IL-8RA was not as prominent as IL-8RB. There was no difference in LVEC expression of IL-8RB when comparing the benign and malignant samples. See figure 1(G-L) for an example of SVEC and LVEC expression of IL-8RA and IL-8RB.

DISCUSSION

Breast cancer is the most common cause of death in women 35-45 years of age. Over 46,000 women in the United States alone die of breast cancer each year[11]. Because breast carcinomas are composed of a heterogeneous mixture of cells with differing growth and metastatic potential, it is hard to predict which cancers will eventually metastasize[12]. Much attention has been given to trying to identify markers that can better predict the growth and metastatic potential of a tumor. Some factors associated with poor outcome include presentation with advanced stage disease, the lack of estrogen receptors, invasion of neural and vascular structures, and tumors that demonstrate a high degree of neovascularity[13-15]. Recently it has become clear that patients whose tumors demonstrate a high degree of neovascularity have a shorter disease free survival rate, and that these patients are more likely to develop distant metastases[16]. Angiogenesis, or the process of formation of new blood vessels, has been shown to be critical for the growth and metastasis of human breast cancer[11]. It is believed that angiogenesis is under the control of chemical signals known as angiogenic factors (AF).

Additionally, tumor cells also have the ability to stimulate their own growth by producing and secreting their own growth factors and responding to these factors via receptor mediated pathways. This autocrine/paracrine pathway promotes uncontrolled tumor proliferation and plays a key role in the metastatic process. Regulating these growth factors and AF is the key to controlling angiogenesis and tumor proliferation. The cytokine IL-8 is both a proven tumor growth factor as well as a potent AF. We feel that this cytokine and its receptors play an important role in breast tumor growth and metastasis.

Interleukin-8 (IL-8) : IL-8 is a member of a family of cytokines that are involved in proinflammatory and reparative processes. This small 8-10 kD cytokine is heparin binding and is produced by many cell types including mononuclear cells, fibroblasts, endothelial cells and keritinocytes[17-18]. IL-8 has been shown to display chemotactic activity both in vivo and in vitro[19-20] and to be an angiogenic factor with its ability to induce proliferation and chemotaxis of endothelial cells[1]. Besides its importance as a chemotactic and angiogenic factor, IL-8 has recently been proven to induce cellular proliferation. IL-8 induced proliferation of human glioma cells[3], keratinocytes[4], and aortic smooth muscle[5] in vitro. Recently, IL-8 has been shown to be present in many neoplastic diseases including melanoma[6], prostate[7], head and neck[8], bronchogenic[9], and colorectal[10] cancer. Interestingly, in many of these instances, increased levels of IL-8 correlated with higher degrees of metastasis or more aggressive less differentiated tumors (i.e. poorer outcome). This further supports the importance of IL-8 and its regulation in the control of many disease states.

We recently have demonstrated the presence of IL-8 on tumor cells and in the surrounding VEC's in HBC tissues. (manuscript in preparation) Furthermore, results from our analysis of breast tumor homogenates has indicated that there is an inverse relationship between IL-8 levels and estrogen receptor levels. (manuscript in preparation) Because higher levels of estrogen receptors are indicative of better outcome, in breast cancer, increased IL-8 levels correlate with poorer prognosis as is also true in the above mentioned disease states. For IL-8 to elicit these physiological effects, it would be essential that IL-8 receptors be expressed on target

cell, i.e. tumor cells and VEC. We set out to verify that the IL-8 receptors do indeed exist on tumor cells and on the VEC's in the tumor microenvironment.

IL-8 receptors - The IL-8 receptors (IL-8RA and IL-8RB) are present on a variety of cell types. Neutrophils, monocytes, keratinocytes, endothelial cells, fibroblasts, macrophages, smooth muscle and T cells have all been shown to express one or both of these receptors. To date, two distinct IL-8 receptors have been isolated, characterized and cloned. These receptors are 77% homologous and are seven transmembrane domain receptors. Both receptors act via G proteins through the phospholipase C pathway which induces the release of intracellular calcium and the activation of protein kinase C[21]. Both IL-8RA and IL-8RB bind IL-8 with high affinity (Kd = 0.1-4.0 nM)[22]. Several other cytokines also bind to only IL-8RB with high affinity. Neutrophil activating protein (NAP-2) and melanoma growth stimulatory activity (MGSA) also known as Gro bind IL-8RB with affinity similar to IL-8, but show a much lower affinity for IL-8RA (Kd=100-130 nM)[22]. Hence while IL-8RA is specific for IL-8 IL-8RB binds a variety of compounds. Chunthoarapai and Kim[22] further investigated the difference in affinities of IL-8RA and IL-8RB for IL-8 on human neutrophils. This group found that the affinity of IL-8RB for IL-8 (Kd=0.031 - 0.133nM) was two to five times greater than the affinity of IL-8RA for IL-8 (Kd=0.096 - 0.168nM).

IL-8, IL-8 receptors, and tumor cells

It is clear from our results that both IL-8RA and IL-8RB are expressed on HBC tumor cells. All of the samples studied revealed the expression of both of these receptors. In the tumor cell environment, this

data supports our hypothesize that IL-8 may be working as a proliferative agent or growth factor. In view of the fact that other known growth factors (MSGA, NAP-2) share an equal affinity and react with the IL-8RB, and that IL-8 definitely causes proliferation in a variety of cell types, IL-8 binding could trigger a pathway of proliferation in the breast cancer tumor environment. The exact role of IL-8RA, which binds only IL-8 with high affinity is not clear. One study discovered that IL-8RA once internalized (after IL-8 binding), is recovered at a much higher rate than IL-8RB[22]. This group speculated that in areas of higher IL-8 concentration (i.e. site of inflammation, or in this case the tumor site) IL-8RA may play a major active role in transmitting the IL-8 signal due to the receptors rapid recovery rate. Whether IL-8RA is signaling proliferation as we propose IL-8RB is doing is not known at this time, but is clear that IL-8RA and IL-8RB are both expressed by breast cancer tumor cells.

IL-8, IL-8 receptors, and angiogenesis

The presence of IL-8RA and IL-8RB on SVEC and LVEC's is critical for the recognition of IL-8 as an angiogenic factor. In our study, there existed a difference of expression between the two receptors, on the SVEC as well as on the LVEC. While IL-8RB was expressed prominently on both SVEC and LVEC, IL-8RA was clearly present on only 25 % of the SVEC and 14% of the LVEC's.

Chuntharapai and Kim[22] studied the different function of IL-8RA and IL-8RB. They concluded that IL-8 has a two to five fold higher affinity for IL-8RB than IL-8RA. They postulated that during the inflammatory response, IL-8 secretion at the site of inflammation diffuses through the tissue to reach blood vessels at a distant site. IL-8RB then receives the signal, (because of its higher affinity and the lower concentration of IL-8 at

the distant site) and initiate the migration of neutrophils towards the site of inflammation. As the neutrophils approached the site of inflammation (i.e. higher concentration of IL-8), IL-8RA would become the dominant receptor in mediating the IL-8 signal. This hypothesis has been supported when looking at IL-8 levels in patients with chronic inflammatory airway disease[23].

A similar scenario can be found in the breast tumor model. In breast cancer, we believe the tumor is producing IL-8 which functions in an autocrine/paracrine manner. This cytokine is diffusing outward, until it reaches a blood vessel at which point it binds to IL-8RB (the prominent receptor on SVEC and LVEC). At that time, the endothelial cells are signaled to start the angiogenesis process. Degradation of the basement membrane of the parent vessel, migration of the endothelial cells towards the angiogenic stimulus (IL-8), proliferation and maturation of the endothelial cells, organization into capillary tubes and finally formation of a new basement membrane occurs as the angiogenic process unfolds[24-25]. This process of angiogenesis is essential for tumor growth and metastasis. We propose that IL-8RB is the dominant receptor on the VEC and is responsible for recognizing the angiogenic factor IL-8.

IL-8, IL-8 receptors, malignant vs. benign tissue:

One of our main objectives in testing benign breast disease specimens was to discover whether the expression of the IL-8 receptors was different in the benign samples when compared to the malignant ones. A comparison of the distribution of IL-8, IL-8RA and IL-8RB in malignant and benign breast disease is shown in Table 2. The ductal epithelial cells in benign breast disease displayed positive expression of IL-8RA in 50% and IL-8RB in 37% of the specimens tested. Considering that all of the tumor

specimens expressed both of the IL-8 receptors, it appears that these receptors are upregulated in the malignant disease state.

Furthermore, when we examined the adjacent areas with normal or hyperplastic ductal formation (in the malignant samples), we were excited to find that these ducts showed a still different staining pattern than the ducts in the benign specimens. For example, in the benign disease samples, while 50% of the ductal epithelial cells expressed IL-8RA, 88% of the normal adjacent ducts (in the malignant samples) expressed this receptor. We found this progression in the expression of IL-8RA from benign ductal epithelial (50%) to normal adjacent ductal epithelial (in malignant) (88%), to malignant tumor cells (100%) to be present. For IL-8RB expression, we found very little difference between the ductal epithelial staining when comparing the benign DEC with the normal adjacent DEC from the malignant specimens.

Another difference was observed when comparing the benign to malignant samples. While the majority of benign ductal myoepithelial cells expressed both IL-8RA and IL-8RB, the expression of IL-8RA on the normal adjacent ductal myoepithelial cells (in malignant samples) was only apparent on 5% of the samples.

The pattern of staining on the VEC in the benign breast samples was comparable to that found in the malignant samples. IL-8RB was expressed on the VEC in the majority of all disease states tested. IL-8RA was expressed in almost all of the SVEC's in the benign samples (88%) while it was less prominent in the malignant samples (65%).

In conclusion, we found that IL-8RA & IL-8RB are expressed by human breast cancer tumors. In addition, we observed IL-8RB to be the dominant receptor expressed by SVEC and LVEC. This data supports our

hypothesis that IL-8 plays an important role as a tumor cell activator (proliferation) and a VEC activator (angiogenesis). Future studies targeting the regulation of IL-8 and its receptors, could prove promising in preventing tumor growth and metastasis in human breast cancer.

METHODOLOGY

Breast tissue specimens: Breast cancer tissue (36 invasive, 7 ductal carcinoma *in situ* (DCIS)) as well as 8 benign breast disease tissue specimens were obtained from archival specimens in the Department of Pathology, University of Connecticut School of Medicine. These formalin fixed paraffin-embedded tissue were cut into 4um sections and mounted on slides for evaluation.

Immunohistochemical techniques: Immunohistochemical analysis of the tissue specimens was performed by indirect immunoperoxidase staining. Briefly, four-micrometer paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in graded alcohol (100%, 95%, 70%, and 50%). To inhibit endogenous peroxidase, the sections were immersed in methyl alcohol containing 0.01% H_2O_2 for 20 minutes. Sections were allowed to air dry and then blocked with 5% normal goat serum in phosphate buffered saline (PBS) pH 7.4, at room temperature (RT) for 1 hour. The sections were then washed 3 times in PBS and a 1/200 dilution of rabbit anti-human IL-8RA or IL-8RB (Santa Cruz, Santa Cruz, Calif) in 5% blocking serum was added and incubated overnight at 4 C. Concurrently, as a control, the primary antibody was preincubated (2hr, at room temperature) with a 10x (by weight) excess of the specific antigen in order to block the reactive sites. This solution was placed on the slides and incubated overnight at 4 C. After the incubation period, biotinylated goat anti-rabbit antibody (Vector, Burlington, Calif.) 1/200 in 5% blocking serum was applied to sections and allowed to incubate for 1 hour at RT. Sections were washed three times in PBS in between the following steps. HRP-Streptavidin (Zymed, San Francisco, Calif.) at a 1/100 dilution in PBS was applied and allowed to incubate for 45 minutes. The sections were

then incubated with 3-amino-9-ethylcarbazine in 0.1-M sodium acetate buffer (pH 5) and 0.03% H_2O_2 for 20 minutes at RT. Sections were then counterstained in Harris' hematoxylin (Sigma) for 2 mins. Slides were then washed in distilled water, dipped in dilute ammonium hydroxide and mounted in crystal mounting solution (Biomeda, Foster City, Calif.).

The stained slides were evaluated. For the malignant diagnoses, (invasive and DCIS) the presence of staining on the tumor cells, VEC, (both small vessels (SVEC) and large vessels (LVEC)), along with the smooth muscle surrounding the arterial large vessels was recorded. In samples where areas of adjacent normal or hyperplastic ductal morphology was present, the staining pattern of these ducts was noted. On the benign specimens, the staining pattern of the ductal epithelial cells (DEC) was noted, as well the staining of the SVEC, LVEC and the surrounding arterial smooth muscle.

When evaluating the VEC, a distinction was made between the SVEC and LVEC. Any vessel containing only endothelial cells (less than 6 cells with no evidence of a smooth muscle layer) was labeled SVEC. Larger vessels, (i.e. veins), or vessels that clearly contained a smooth muscle layer (i.e. arteries) were labeled LVEC.

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FIGURE LEGEND

Figure 1

Expression of IL-8RA and IL-8RB by Tumor Cells, Benign Ductal Epithelial Cells (DEC) and Vascular Endothelial Cells (VEC) [Both Small Vessel Endothelial Cells (SVEC) and Large Vessel Endothelial Cells (LVEC)]. A) tumor cell expression of IL-8RA (40x), B) tumor cell expression of IL-8RB (40x), C) tumor cell control (40x), D) benign DEC expression of IL-8RA, [note apical staining pattern] (20x), E) benign DEC expression of IL-8RB (20x), F) benign DEC control (20x), G) SVEC expression of IL-8RA (40x), H) SVEC expression of IL-8RB (40x), I) SVEC control (40x), J) LVEC expression of IL-8RA, [note negative endothelial cells and positive smooth muscle] (40x), K) LVEC expression of IL-8RB, [note positive endothelial cell and smooth muscle] (40x), L) LVEC control (40x).

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Expression of Interleukin-8 Receptors on Tumor Cells and Vascular Endothelial Cells in Human Breast Cancer Tissue

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Abstract. Background: *Recently, we demonstrated the presence of Interleukin-8 (IL-8) in human breast cancer (HBC) tissue. We hypothesize that the IL-8 receptors are present and play a role in tumor cell and vascular endothelial cell (VEC) activation (e.g. proliferation and angiogenesis). Materials and Methods: Immunohistochemical analysis for IL-8 receptors (IL-8RA and IL-8RB) was performed on 43 malignant and 8 benign breast tissue samples. Results: Tumor cells expressed IL-8RA and IL-8RB in all of the malignant specimens. Only 50% of the benign ductal epithelial cell (DEC) samples expressed these receptors. The majority of small vessel endothelial cells (SVEC) expressed IL-8RA and IL-8RB, while large vessel endothelial cells (LVEC) showed primarily IL-8RB expression. Conclusions: Our results demonstrate that tumor and VEC express the IL-8 receptors and likely play a role in regulating tumor and VEC activation which controls proliferation, angiogenesis and metastasis in HBC.*

Recently the cytokine Interleukin 8 (IL-8) has been shown to be both a potent angiogenic factor (AF) and a growth factor in both normal and disease states. For example, IL-8 has been proven to stimulate angiogenesis in the normally avascular environment of the cornea (1). Additionally, IL-8 has been shown to be present in many neoplastic diseases including in cultured melanoma cells (2) and in prostate (3) and head and neck cancers (4). Previously we have reported IL-8 expression on tumor cells and on the VEC in human breast cancer tissue (Kurtzman *et al*, Abstract presented at Society of Surgical Oncology, March 1994). For the functional expression of IL-8 to occur, both IL-8 and the IL-8 receptors must be present within the tumor environment.

Currently, nothing is known regarding the expression of the IL-8 receptors (IL-8RA and IL-8RB) in breast cancer or in any other neoplastic disease. For the present study, we hypothesized that IL-8 receptors are expressed on the tumor cells as well as on the VEC ,within the tumor microenvironment, in human breast cancer and likely contribute to tumor growth and angiogenesis. To test this hypothesis, we determined the distribution of the two IL-8 receptors, IL-8RA and IL-8RB, on cells in tissue from malignant and benign human breast disease patients using immunohistochemical techniques. Our studies clearly demonstrate that IL-8RA and IL-8RB are present on both tumor cells and VEC, and support our hypothesis on the role of the IL-8 system in human breast cancer.

Materials and Methods

Breast tissue specimens. Breast cancer tissue [36 invasive, 7 ductal carcinoma In situ (DCIS)] as well as 8 benign breast disease tissue specimens were obtained from archival specimens in the Department of Pathology, University of Connecticut School of Medicine. These formalin fixed paraffin-embedded tissue were cut into 4mm sections and mounted on slides for evaluation.

Immunohistochemical techniques. Immunohistochemical analysis of the tissue specimens was performed by indirect immunoperoxidase staining as previously described (4). Anti human IL 8 receptor A and B (IL-8RA, IL-8RB) were obtained from Santa Cruz Biotechnologies, (Santa Cruz, CA). For all of the immunohistochemical analysis performed in this study, a set of controls was run to verify the specificity of the antibody reaction. All of the control slides were negative, and showed no evidence of staining. Figure 1 (C,F,I,L) shows an example of these control slides.

The stained slides were evaluated. For the malignant diagnoses, (invasive and DCIS) the presence of staining on the tumor cells, VEC, [both small vessels (SVEC) and large vessels (LVEC)], along with the smooth muscle surrounding the arterial large vessels was recorded. In samples where areas of adjacent normal or hyperplastic ductal morphology was present, the staining pattern of these ducts was noted. On the benign specimens, the staining pattern of the ductal epithelial cells (DEC) was noted, as well the staining of the SVEC, LVEC and the surrounding arterial smooth muscle.

When evaluating the VEC, a distinction was made between the SVEC and LVEC. Any vessel containing only endothelial cells (less than 6 cells

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Key Words: Interleukin -8 (IL-8), IL-8 receptors, human breast cancer, tumor proliferation, angiogenesis.

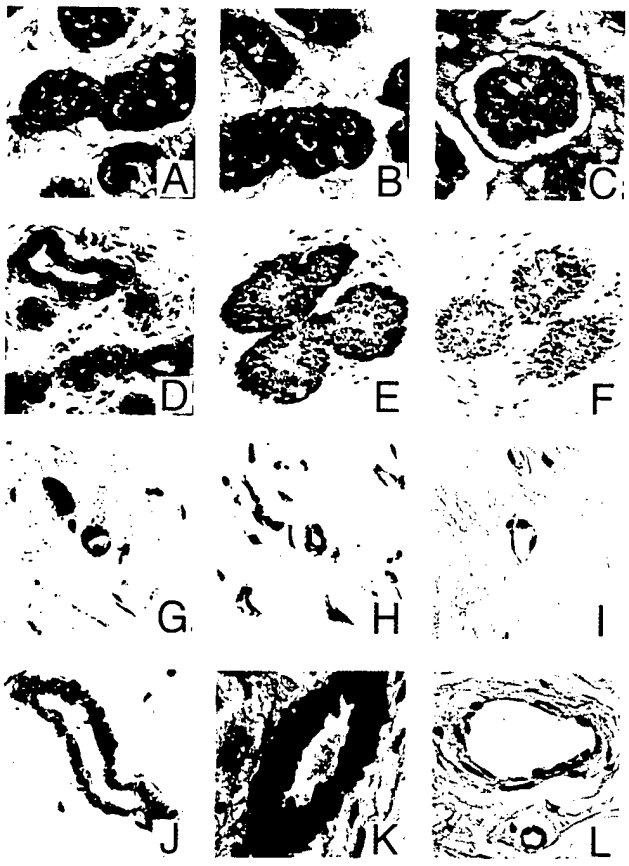

Figure 1. *Expression of IL-8RA and IL-8RB by Tumor Cells, Benign Ductal Epithelial Cells (DEC) and Vascular Endothelial Cells (VEC) [Both Small Vessel Endothelial Cells (SVEC) and Large Vessel Endothelial Cells (LVEC)]. A) tumor cell expression of IL-8RA (40x), B) tumor cell expression of IL-8RB (40x), C) tumor cell control (40x), D) benign DEC expression of IL-8RA, [note apical staining pattern] (20x), E) benign DEC expression of IL-8RB (20x), F) benign DEC control (20x), G) SVEC expression of IL-8RA (40x), H) SVEC expression of IL-8RB (40x), I) SVEC control (40x), J) LVEC expression of IL-8RA, [note negative endothelial cells and positive smooth muscle] (40x), K) LVEC expression of IL-8RB, [note positive endothelial cell and smooth muscle] (40x), L) LVEC control (40x).*

with no evidence of a smooth muscle layer) was labeled SVEC. Larger vessels, (> 6 VEC), or vessels that clearly contained a smooth muscle layer (*i.e.* arteries) were labeled LVEC.

Results

Malignant tumor cell IL-8RA expression. All of the invasive (36/36) and DCIS (7/7) HBC specimens stained positively for the IL-8RA receptor on tumor cells. (Figure 1A) Interestingly, IL-8RA expression showed a wide degree of variation of staining within a tumor, *i.e.* staining tended to be heterogeneous, associated with individual cells or within groups of tumor cells. In well differentiated tumors, with abortive duct formation, staining was in an apical, circular pattern. A number of malignant specimens examined contained areas of normal or hyperplastic ductal morphology adjacent to the tumor. The majority of these areas expressed the IL-8RA on the ductal epithelial cells. Again, as seen in the malignant samples, staining was in an apical pattern, with intense staining closest to the lumen of the duct. The myoepithelial cells surrounding the ductal epithelial cells showed little evidence of IL-8RA expression.

Malignant tumor cell IL-8RB expression. All invasive (36/36) and DCIS (7/7,) HBC specimens stained positively for the IL-8RB receptor on tumor cells. (Figure 1B) Unlike the heterogeneous, cell associated staining displayed for IL-8RA, the staining pattern for IL-8RB was homogeneous throughout the tumor with only slight variations within a specimen. On samples where areas of normal or hyperplastic ductal morphology was present adjacent to the tumor, less than half of the samples showed IL-8RB expression on the ductal epithelial cells. When staining in the non-neoplastic specimens was present, it was not in the apical pattern apparent for IL-8RA expression. Almost all of the myoepithelial cells expressed the IL-8RB. In summary, all of the HBC specimens examined showed that tumor cells express both the IL-8RA and IL-8RB. These results

Table I. *Expression of IL-8 receptors in malignant and benign breast tissue.*

	IL-8RA		IL-8RB	
	POS[1]	%POS[2]	POS[1]	%POS[2]
Malignant				
Tumor cells				
Invasive	36/36	100	36/36	100
DCIS	7/7	100	7/7	100
Total	43/43	100	43/43	100
SVEC				
Invasive	24/36	6 7	34/36	94
DCIS	4/7	57	7/7	100
Total	28/43	65	41/43	95
LVEC				
Invasive	6/36	17	26/36	72
DCIS	0/7	0	4/7	57
Total	6/43	14	30/43	70
Benign breast disease				
Ductal cells				
Epithelial	4/8	50	3/8	37
Myoepithelial	7/8	88	8/8	100
SVEC	7/8	88	7/8	88
LVEC	1/8	13	6/8	75

1. POS: # positive / total
2. % POS: percent positive

Table II. *Comparison of IL-8, IL-8RA and, IL-8RB expression in malignant and benign breast tissue.*

Tissue	IL-8	IL-8RA	IL-8RB
Malignant			
Tumor Cells	+++	+++	+++
SVEC	+++	++	+++
LVEC	+++		++
Benign breast disease			
Ductal epithelial cells	+++	+	+
SVEC	+++	++	+++
LVEC	---	---	++

Immunohisochemical scoring:
+++ >90 % of positive specimens
++ 51-90 % positive specimens
+ 21-50 % positive specimens
--- < 20 % positive specimens

demonstrate that if IL-8 is indeed acting as a growth factor, it can be recognized by the tumor cells in HBC. Figure 1 (A-B) displays the tumor cell expression of IL-8RA and IL-8RB.

Malignant SVEC IL-8RA expression. In the invasive HBC, 67% (24/36) and in DCIS, 57% (4/7) of the samples showed some positive staining on the SVEC for IL-8RA. (Figure 1G) Overall, or 65% (28/43) of the malignant HBC patients showed IL-8RA expression. Even though positive SVEC's were located in 28 of the slides, only 11 (25%) had SVEC staining that was clearly evident while the remainder showed areas of positive and negative SVEC staining.

Malignant SVEC IL-8RB expression. A far different picture was present for IL-8RB expression on SVEC. (Figure 1H) In the invasive samples, 94% (34/36) and 100% (7/7) of DCIS, were positive for IL-8RB expression. A total of 95% (41/43) of the malignant specimens showed positive staining for this receptor. The SVEC's were very visible and easy to locate in the majority of slides.

Malignant LVEC IL-8RA expression. In the invasive samples only 17% (6/36) while none of the DCIS specimens showed expression of IL-8RA on the LVEC. Overall, 14% (6/43) of the malignant samples showed positive staining for IL-8RA. Figure lJ shows the typical negative staining of the LVEC, while the smooth muscle surrounding the arterial vessels consistently expressed IL-8RA.

Malignant LVEC IL-8RB expression. The expression of IL-8RB on the LVEC was distinctly different from that of IL-8RA.72% (26/36) of the invasive and 57 % (4/7) of the DCIS, for a total of 70% (30/43) of the malignant samples showed positive staining for IL-8RB. The smooth muscle surrounding the arterial large vessels stained intensely for IL-8RB in the majority of the malignant specimens studied. From our investigation of the expression of IL-8RA and IL-8RB by the VEC, it is evident that while both IL-8RA and IL-8RB can be expressed, IL-8RB is the prominent receptor present on the SVEC and LVEC. An example of SVEC and LVEC expression of IL-8RA and IL-8RB can be seen in Figure 1 (G,H,J,K)

Benign DEC IL-8RA expression. Expression of IL-8RA on the ductal epithelial cells occurred in 50% (4/8) of the specimens. All of these displayed staining in an apical pattern, on the portion of the cell closest to the lumen of the duct. Eighty-eight percent (7/8) showed IL-8RA expression on the myoepithelial cells surrounding the ductal epithelium.

Benign DEC IL-8RB expression. Only 37% (3/8) of the samples tested stained for IL-8RB on the ductal epithelium. Two of the three showed the apical pattern of staining evident with IL-8RA expression. All (8/8) of the specimens showed expression of IL-8RB on the myoepithelial cells. In summary,

only about 50% of the benign DEC samples expressed IL-8RA or IL-8RB, whereas the majority of the myoepithelial cells surrounding the ducts expressed these receptors. Expression of IL-8RA and IL-8RB by the ductal epithelial cells is displayed in Fgure 1 (D-E).

Benign SVEC IL-8RA and IL-8RB expression. The majority of the benign breast disease samples (88%) showed positive staining for both IL-8RA and IL-8RB. As in the malignant samples, most of the samples showed positive and negative areas of SVEC IL-8RA expression, while IL-8RB expression by SVEC's was clearly present throughout the specimens.

Benign LVEC IL-8RA and IL-8RB expression. Only 13% (1/8) of the samples tested were positive for IL-8RA on the LVEC in the benign breast samples whereas the majority, 75% (6/8), were positive for IL-8RB. The smooth muscle surrounding the LVEC stained positively in 63% (5/8) of the samples tested for both of the receptors. In summary, the majority of SVEC's expressed both IL-8RA and IL-8RB. As was seen in the malignant samples, IL-8RA was not as prominent as IL-8RB. There was no difference in LVEC expression of IL-8RB when comparing the benign and malignant samples. See Figure 1(G-L) for an example of SVEC and LVEC expression of IL-8RA and IL-8RB. The results cited above are summarized in Table I.

Discussion

Angiogenesis, or the process of formation of new blood vessels, has been shown to be critical for the growth and metastasis of human breast cancer (5). It is believed that angiogenesis is under the control of chemical signals known as angiogenic factors (AF). Additionally, tumor cells also have the ability to stimulate their own growth by producing and secreting their own growth factors and responding to these factors *via* receptor mediated pathways. This autocrine/paracrine pathway promotes uncontrolled tumor proliferation and plays a key role in the metastatic process. Regulating these growth factors and AF is the key to controlling angiogenesis and tumor proliferation. The cytokine IL-8 is both a proven tumor growth factor as well as a potent AF. We feel that this cytokine and its receptors play an important role in breast tumor growth and metastasis.

We recently have demonstrated the presence of IL-8 on tumor cells and in the surrounding VEC's in HBC tissues (manuscript in preparation). Furthermore, results from our analysis of breast tumor homogenates has indicated that there is an inverse relationship between IL-8 levels and estrogen receptor levels (manuscript in preparation). Because higher levels of estrogen receptors are indicative of better outcome, in breast cancer, increased IL-8 levels correlate with poorer prognosis. For IL-8 to elicit these physiological effects, it would be essential that IL-8 receptors be expressed on target cell, *i.e.* tumor cells and VEC. We set out to verify that the IL-8 receptors do indeed exist on tumor cells and on the VEC's in the tumor microenvironment.

The IL-8 receptors (IL-8RA and IL-8RB) are present on a variety of cell types. Neutrophils, monocytes, keratinocytes, endothelial cells, fibroblasts, macrophages, smooth muscle and T cells have all been shown to express one or both of these receptors. To date, two distinct IL-8 receptors have been isolated, characterized and cloned. These receptors are 77% homologous and are seven transmembrane domain receptors. Both receptors act *via* G proteins through the phospholipase C pathway which induces the release of intracellular calcium and the activation of protein kinase C (6). Both IL-8RA and IL-8RB bind IL-8 with high affinity (Kd = 0.1- 4.0 nM). Several other cytokines also bind to only IL-8RB with high affinity. Neutrophil activating protein (NAP-2) and melanoma growth stimulatory activity (MGSA) also known as Gro bind IL-8RB with affinity similar to IL-8, but show a much lower affinity for IL-8RA (Kd=100-130 nM)(7). Hence, while IL-8RA is specific for IL-8 IL-8RB binds a variety of compounds. Chunthoarapai and Kim (7) further investigated the difference in affinities of IL-8RA and IL-8RB for IL-8 on human neutrophils. This group found that the affinity of IL-8RB for IL-8 (Kd=0.031-0.133nM) was two to five times greater than the affinity of IL-8RA for IL-8 (Kd=0.09'6-0. 1 68nM).

IL-8, IL-8 receptors, and tumor cells. It is clear from our results that both IL-8RA and IL-8RB are expressed on HBC tumor cells. All of the samples studied revealed the expression of both of these receptors. In the tumor cell environment, this data supports our hypothesize that IL-8 may be working as a proliferative agent or growth factor. In view of the fact that other known growth factors (MSGA, NAP-2) share an equal affinity and react with the IL-8RB, and that IL-8 definitely causes proliferation in a variety of cell types, IL-8 binding could trigger a pathway of proliferation in the breast cancer tumor environment. The exact role of IL-8RA, which binds only IL-8 with high affinity is not clear. One study discovered that IL-8RA once internalized (after IL-8 binding), is recovered at a much higher rate than IL-8RB (7). This group speculated that in areas of higher IL-8 concentration (*i.e.* site of inflammation, or in this case the tumor site) IL-8RA may play a major active role in transmitting the IL-8 signal due to the receptors rapid recovery rate. Whether IL-8RA is signaling proliferation as we propose IL-8RB is doing is not known at this time, but is clear that IL-8RA and IL-8RB are both expressed by breast cancer tumor cells.

IL-8, IL-8 receptors, and angiogenesis. The presence of IL-8RA and IL-8RB on SVEC and LVEC's is critical for the recognition of IL-8 as an angiogenic factor. In our study, there existed a difference of expression between the two receptors, on the SVEC as well as on the LVEC. While IL-8RB was expressed prominently on both SVEC and LVEC,

IL-8RA was clearly present on only 25 % of the SVEC and 14% of the LVEC's.

Chuntharapai and Kim (7) studied the different function of IL-8RA and IL-8RB. They concluded that IL-8 has a two to five fold higher affinity for IL-8RB than IL-8RA. They postulated that during the inflammatory response, IL-8 secretion at the site of inflammation diffuses through the tissue to reach blood vessels at a distant site. IL-8RB then receives the signal, (because of its higher affinity and the lower concentration of IL-8 at the distant site) and initiates the migration of neutrophils towards the site of inflammation. As the neutrophils approached the site of inflammation (*i.e.* higher concentration of IL-8), IL-8RA would become the dominant receptor in mediating the IL-8 signal. This hypothesis has been supported when looking at IL-8 levels in patients with chronic inflammatory airway disease (8).

A similar scenario can be found in the breast tumor model. In breast cancer, we believe the tumor is producing IL-8 which functions in an autocrine/paracrine manner. This cytokine is diffusing outward, until it reaches a blood vessel at which point it binds to IL-8RB (the prominent receptor on SVEC and LVEC). At that time, the endothelial cells are signaled to start the angiogenesis process. Degradation of the basement membrane of the parent vessel, migration of the endothelial cells towards the angiogenic stimulus (IL-8), proliferation and maturation of the endothelial cells, organization into capillary tubes and finally formation of a new basement membrane occurs as the angiogenic process unfolds (9,10). This process of angiogenesis is essential for tumor growth and metastasis. We propose that IL-8RB is the dominant receptor on the VEC and is responsible for recognizing the angiogenic factor IL-8.

IL-8, IL-8 receptors, malignant vs. benign tissue. One of our main objectives in testing benign breast disease specimens was to discover whether the expression of the IL-8 receptors was different in the benign samples when compared to the malignant ones. A comparison of the distribution of IL-8, IL-8RA and IL-8RB in malignant and benign breast disease is shown in Table II. The ductal epithelial cells in benign breast disease displayed positive expression of IL-8RA in 50% and IL-8RB in 37% of the specimens tested. Considering that all of the tumor specimens expressed both of the IL-8 receptors, it appears that these receptors are upregulated in the malignant disease state.

Furthermore, when we examined the adjacent areas with normal or hyperplastic ductal formation (in the malignant samples), we were excited to find that these ducts showed a still different staining pattern than the ducts in the benign specimens. For example, in the benign disease samples, while 50% of the ductal epithelial cells expressed IL-8RA, 88% of the normal adjacent ducts (in the malignant samples) expressed this receptor. We found this progression in the expression of IL-8RA from benign ductal epithelial (50%) to normal adjacent ductal epithelial (in malignant) (88%), to malignant tumor cells (100%) to be present. For IL-8RB expression, we found very little difference between the ductal epithelial staining when comparing the benign DEC with the normal adjacent DEC from the malignant specimens.

Another difference was observed when comparing the benign to malignant samples. While the majority of benign ductal myoepithelial cells expressed both IL-8RA and IL-8RB, the expression of IL-8RA on the normal adjacent ductal myoepithelial cells (in malignant samples) was only apparent on 5% of the samples.

The pattern of staining on the VEC in the benign breast samples was comparable to that found in the malignant samples. IL-8RB was expressed on the VEC in the majority of all disease states tested. IL-8RA was expressed in almost all of the SVEC's in the benign samples (88%) while it was less prominent in the malignant samples (65%).

In conclusion, we found that IL-8RA and IL-8RB are expressed by human breast cancer tumors. Furthermore, there is increased expression of these receptors in malignant breast tissue. In addition, we observed IL-8RB to be the dominant receptor expressed by SVEC and LVEC. This data supports our hypothesis that IL-8 plays an important role as a tumor cell activator (proliferation) and a VEC activator (angiogenesis). Future studies targeting the regulation of IL-8 and its receptors, could prove promising in preventing tumor growth and metastasis in human breast cancer.

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CYTOKINES IN HUMAN BREAST CANCER: IL-1 ALPHA AND IL-1 BETA EXPRESSION

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ABSTRACT

We hypothesize that Interleukin 1α (IL-1α) and Interleukin 1β (IL-1β) are present and tumor cell associated in human breast cancer (HBC) specimens. To test our hypothesis: 1) immunologic analysis was performed on HBC histologic sections for IL-1α and IL-1β distribution; and 2) homogenates of HBC tumors were analyzed for levels of IL-1α, IL-1β and Interleukin 8 (IL-8) expression. Immunohistochemical analysis demonstrated the presence of IL-1α and IL-1β in tumor cells in patients with invasive cancer and ductal carcinoma *in situ* (DCIS). Quantitative analysis confirmed the presence and positive correlation of IL-1α and IL-1β to IL-8, a known angiogenic factor, in cancer specimens. These studies demonstrate that tumor-associated IL-1α, IL-1β are present and in the tumor microenvironment and may play a pivotal role in regulating breast tumor growth and metastasis.

INTRODUCTION

Human breast cancers (HBC) are histologically characterized by tumor cell proliferation and extensive new vessel ingrowth (angiogenesis) (1, 2). Tumor cell proliferation and new vessel formation within tumor tissue is generally believed to be controlled by a variety of locally elaborated factors including cytokines (*e.g.*, growth and angiogenic factors) (3-7). Cytokines are low molecular weight glycoproteins secreted by tissue, inflammatory, immunologic and tumor cells, that can regulate cell function in both an autocrine or paracrine fashion. Cytokines, such as interleukin 8 (IL-8), basic Fibroblast Growth Factor (bFGF) and Vascular Endothelial Growth Factor (VEGF) are recognized angiogenic factors. Previous studies in inflammation and trauma have demonstrated *in vitro* that angiogenic factors such as IL-8 and VEGF can be induced by specific cytokines such as IL-1 and Tumor Necrosis Factor (TNF) (8). These observations raise the question of whether IL-1 or TNF may control the expression of IL-8 and other pro-tumorigenic cytokines within the tumor microenvironment (TME) of breast cancer. Recently, our lab has demonstrated that IL-1 stimulates the production of IL-8 in primary cell cultures of HBC, suggesting that IL-1 may be a pivotal cytokine controlling the angiogenesis seen in breast cancer (9). Interestingly, few studies have investigated the presence, distribution or levels of IL-1 in cancer. These observations have led us to the general hypothesis that: 1) IL-1 cytokines (IL-1α and IL-1β) are present in the HBC TME, 2) that IL-1α and IL-1β are produced by tumor cells, and 3) that the local production of IL-1 within the tumor environment is pivotal in controlling the expression of protumorigenic factors such as IL-8. Since we hypothesize that IL-1 is an important regulator of the expression of pro-tumorigenic factors such as IL-8 in the TME, it is essential that we

demonstrate of the presence, distribution, levels and correlation of IL-1 family and IL-8 in HBC tissue as the first step in understand IL-1's biologic function in HBC.

MATERIALS AND METHODS

Patient Population

Patients for this study represent individuals who underwent surgical procedures for benign and malignant breast disorders at the John Dempsey Hospital (Farmington, CT) and Saint Francis Medical Center (Hartford, CT).

Tumor Specimens

Patient specimens used for immunohistochemistry were identified by searching the Department of Pathology databases. The paraffin tissue blocks from these patients were obtained, and used for the immunohistochemical studies described below. Fresh tumor tissue obtained from patients undergoing surgery (either lumpectomy or mastectomy) for breast cancer were also used in this study to determine tissue levels of the various cytokines. IL-1α, IL-1β, IL-8 and protein analysis was also performed on the tumor tissue homogenates (see below).

Immunohistochemical Analysis

Tissue was embedded in paraffin and processed into 4-6 μm sections. Paraffin-imbedded sections were then deparaffinized in xylene and rehydrated in graded alcohol (100%, 95%, 75% and 50%). Samples were immersed in methanol containing 0.01% hydrogen peroxide (H_2O_2) for 20 minutes for inhibition of endogenous peroxidases, allowed to air dry, and then blocked with 5% normal rabbit serum (Vector, Burlington, CA) in PBS at room temperature (RT) for 1 hour. The resulting sections were then

washed three times with PBS (pH 7.4). Next, primary antibodies (IL-1α 1:500 dilution, IL-1β 1:600 dilution (Santa Cruz Laboratories, Santa Cruz, CA) in blocking serum were added to the slides. As a specificity control, the primary antibodies were preincubated (2 hrs at RT) with a 20X (by weight) excess of appropriate antigens prior to addition to the histologic sections. Following overnight incubation, biotinylated rabbit anti-goat IgG (Vector, Burlington, CA) was applied to the sections and allowed to incubate for one hour at RT. HRP-streptavidin (Zymed, San Francisco, CA) at a 1:100 dilution in PBS was applied to the sections and incubated at RT for 1 hour. The sections were incubated sequentially with 3-amino-9-ethyl carbazole (AEC) in 0.1 molar sodium acetate buffer (pH 5.0) and 0.03 percent H_2O_2 for 20 minutes at RT. Samples were then counter-stained with Mayer's hematoxylin (Sigma, St. Louis, MO) for 3 minutes, washed extensively in distilled H_20, and dipped in dilute ammonium hydroxide. Sections were then mounted in crystal mounting solution (Biomedia, Foster City, CA) and analyzed by light microscopy.

The immunohistochemical analysis was performed by two of the authors. Staining was graded from 0-4+, 0 = no staining; 1+ = faint staining; 2+ = in-homogeneous staining; 3+ = homogeneous staining; and 4+= intense staining. Any staining (1+ or higher) was considered positive (see Tables 1 and 3).

Tissue Homogenates

Tumor tissue samples were stored in a -70°C freezer until processing. They were then placed in 2.5 ml of phosphate buffered saline (PBS) and homogenized in a tissue homogenizer. The resulting homogenate were centrifuged at 4°C at 40,000 RPM for 30 minutes. The supernatants were then aliquoted and frozen at -70°C.

Cytokine Analysis

Tumor tissue homogenates were initially evaluated for total protein (TP). Immunoassays of tumor homogenates were performed using IL-1α and IL-1β enzyme linked immunoabsorbant assay (ELISA) that was developed in this laboratory using matched antibody pairs and cytokine standards (Endogen, Cambridge, MA). Briefly, for each assay, monoclonal anti IL-1α or IL-1β antibody was coated onto a microtiter plate, and incubated overnight at RT. Unbound antibody was washed away and a blocking solution (PBS with 4% BSA pH 7.2) was added to the plate and incubated for 1 hour at RT. The blocking solution was washed away and samples or standards were added to the multiwell plates. All assays were done in duplicate. For the IL-1α assay, the samples were incubated at RT for 1 hour and then secondary polyclonal anti IL-1α antibody was added, and the combination incubated for 1 hour. For the IL-1β assay, the secondary polyclonal antibody was added simultaneously with the samples or standards, and incubated at RT for 2 hours. Following a wash step to remove any unbound sample, standard or antibody, HRP-streptavidin (Zymed, San Francisco, CA) at a 1:8000 dilution in PBS 4% BSA was added and incubated for 30 minutes at RT. Unbound HRP-streptavidin was washed away, and TMB substrate (Dako, Carpinteria, CA) was added to the wells and color developed in proportion to the amount of IL-1α or IL-1β in the sample. The color development was stopped with 4N H_2SO_4 and the plate read at 450 nm. A standard curve was used to determine the quantity of cytokine in the sample. All results were expressed as picograms per milligram total protein.

IL-8 levels were quantified using RIA methods as previously described (10). Standard curves were used to determine the quantity of cytokine in the sample based on the level of radioactivity of each sample. All samples were assayed in duplicate. Results were expressed as the number of picograms of IL-8 per milligram of TP.

Statistical Analysis

The immunohistochemical staining was analyzed by comparing the mean grade for each group (invasive breast cancer, DCIS, benign) using the one-way ANOVA analysis from the JMP 3.0 statistical package (SAS, Cary, NC). A linear regression analysis was also run to ascertain if a relationship existed between the staining for IL-1α and IL-1β for each patient.

To analyze the cytokine levels in the tumor homogenates, the data was transformed into natural log to achieve a normal distribution. The transformed values were then analyzed using a linear regression model (JMP Software). A p value of <0.05 was considered statistically significant.

RESULTS

Histopathologic Correlations

In the present study, we examined 49 breast specimens for IL-1α presence and distribution. Of these, 32 were from patients with invasive breast cancer, 10 were from patients with non-invasive (DCIS) ductal cancer, and 7 were from patients with non-atypical proliferative breast diseases. In addition, we examined 42 breast specimens for IL-1β presence and distribution. Of these, 26 were from patients with invasive breast cancer, 9 were from patients with non-invasive (DCIS) ductal cancer, and 7 were from patients with non-atypical proliferative breast diseases. Within these groups, 17/42 IBC samples, 7/11 DCIS, and 5/9 benign samples were examined for both IL-1α and IL-1β.

IMMUNOHISTOCHEMISTRY ANALYSIS

Interleukin-1-alpha Immunohistochemistry Analysis

Initially, we determined the distribution of IL-1α within the tumor microenvironment of IBC, DCIS, and benign breast disease specimens. Our initial observations indicated that IL-1α was consistently expressed in the tumor cells of 100% of specimens from patients with both IBC and DCIS (see Figure 1 and Table 1). We observed that IL-1α staining pattern seen in all the various cell populations displayed cytoplasmic staining suggesting that IL-1 is likely being produced by the cells, rather than found on cell surface receptors. When histologically normal ducts within the specimens were examined, we saw that normal ductal epithelial cells also appeared consistently IL-1α positive (Table 1: 100% IBC, 90% DCIS, and 100% benign). In IBC, only 9 specimens contained histologically normal ducts. Although all specimens stained to some degree for IL-1α, the intensity of the IL-1α staining differed when comparing the benign and malignant tissues. The intensity of the staining of the tumor cells in the IBC and DCIS specimens was significantly higher than the staining of the ductal cells in the benign breast samples (Table 2: IBC: 2.5 ± 0.11 and DCIS: 2.5 ± 0.22; benign: 1.5 ± 0.36) ($p<0.002$, $p<0.006$, respectively).

Analysis of IL-1α expression in small vessels endothelium within the specimens indicated that IL-1α was consistently expressed in the benign (71%) and DCIS (67%) specimens, as well as the invasive breast cancer 71%. Interestingly, IL-1α expression in the large vessel endothelium of the same specimens demonstrated that in benign disease there was a similar occurrence of IL-1α staining (57%) compared to the small vessel endothelium (71%). However, the occurrence of IL-1α staining of large vessel endothelium in the tumor specimens was dramatically lower when compared to IL-1α staining of the small vessel endothelium in the same specimens [IBC: 39% (large) vs. 71% (small); DCIS: 30% (large) vs. 67% (small)].

Further analysis of the large blood vessels demonstrated that there was consistent expression of IL-1α in the smooth muscle cells surrounding large vessels in all specimens, but slightly diminished expression in the smooth muscle cells associated with invasive breast cancer (IBC: 50%, DCIS: 60%, benign: 71%). Finally, analysis of the fibroblasts within the stroma of the specimens indicated that there was IL-1α staining of fibroblasts in all three types of breast specimens. Thus, these data support our hypothesis that tumor cells likely represent a major and consistent source of IL-1α in the tumor microenvironment, and that other cells such as vascular endothelial cells also can contribute to IL-1α levels within the TME.

Interleukin-1-beta Immunohistochemistry Analysis

Immunohistochemical analysis of the human breast specimens for IL-1β indicated that tumor cells showed a consistent staining pattern for IL-1β (Figure 1 and Table 3: IBC 88%, DCIS 78%). The staining was found to be cytoplasm associated. Analysis of normal ducts found in the specimens indicated that ductal epithelium in IBC, DCIS, and benign samples showed similar consistent levels of positive staining for IL-1β (see Table 3). The intensity of staining of the tumor cells in IBC and DCIS was significantly higher than the intensity of staining of the normal ductal cells in the benign breast samples (Table 2: IBC: 1.86 ± 0.20 and DCIS: 2.11 ± 0.32; benign: 0.75 ± 0.25) ($p<0.040$, $p<0.025$, respectively). Interestingly, when the tissue was further evaluated for IL-1β expression , the small vessel endothelium associated with invasive breast cancer demonstrated more frequent staining (81%) when compared to either DCIS or benign (i.e., 44% and 57%, respectively).

Analysis of IL-1β staining in the large vessels of all three groups, demonstrated staining in fewer of the specimens (range: 11-29%) when compared to the small

vessel endothelium in the same specimens (range: 44-81%). Analysis of the smooth muscle cells associated with vessels, also showed an interesting pattern in which smooth muscle cells associated with invasive cancer had a consistently higher rate of staining (72% staining) when compared to either DCIS (33%) or benign (43%).

Finally, IL-1β analysis of fibroblasts in breast cancer specimens indicated that approximately 40% of specimen stained in IBC and DCIS for IL-1β, but fibroblasts present in the benign breast disease did not show any staining for IL-1β. Thus, these data suggest that the tumor cells may be a major source of IL-1β in breast tissue, as was the case for IL-1α. In addition to the tumor cells, a number of other cells in the tumor microenvironment also express IL-1β antigen.

CYTOKINE QUANTITATION

Interleukin-1-alpha Quantitation

Analysis of breast tumor homogenates indicated that 71 of 82 cancer specimens had detectable levels of IL-1α (i.e. 5 pg./ml). The levels of IL-1α ranged from 0.94 pg./mg TP to 236.20 pg./mg TP, with a mean for the 82 samples of 17.6 ± 3.90 pg./mg TP.

Interleukin-1-beta Quantitation

Analysis of tumor homogenates for IL-1β antigen indicated that 96 of 101 cancer specimens had detectable levels of IL-1β (i.e., > 5 pg./ml). The IL-1β levels were corrected for mg total protein. The IL-1β levels ranged from 0.67 pg./mg TP to 96.3 pg./mg TP. The mean IL-1β value for the 101 samples was 12.02 ± 1.42 pg./mg TP.

Interleukin-8 Quantitation

Analysis of tumor homogenates for IL-8 antigen indicated that 72 of 103 cancer specimens had detectable levels of IL-8 (i.e. > 40 pg./ml) ranging from 4.6 pg./mg TP to 3175.7 pg./mg TP. The mean IL-8 value for the 103 samples was 106.43 ± 38.72 pg./mg TP.

Interleukin-1α, Interleukin-1β and Interleukin-8 Correlations

The levels of IL-1α in the tissue homogenates were compared with levels of IL-8, and IL-1β. Interestingly, we found that IL-1α levels correlated directly with levels of IL-8 (Table 4: $p < 0.01$), a know angiogenic factor, but IL-1α levels did not correlate with IL-1β levels in the homogenates. The levels of IL-1β directly correlated with levels of IL-8 (Table 4 : $p < 0.01$).

DISCUSSION

It has previously been suggested that wounds and tumors share so many common features (*e.g.* cell proliferating angiogenesis, fibrosis, fibrin deposition, etc.) that tumors can be viewed as wounds that will not heal (11). We have extended this concept to investigate the role of cytokines, specifically, the IL-1 family of cytokines that are involved in wound healing, and may be involved in tumor growth and metastasis in HBC.

Cytokines have been implicated as important regulators of cell function in a variety of diseases. For example, cytokines have been considered to be key regulators of tissue cells and leukocytes in chronic inflammatory diseases such as rheumatoid arthritis (1, 12-17), inflammatory bowel disease(18, 19), and interstitial lung disease (20) to name but a few. It has been further postulated that in the case

of acute and chronic inflammation, cytokines such as IL-1 and TNF are present in the tissue microenvironment in quantities sufficient to control both the inflammatory and repair processes. Thus, the important role of cytokines in general, and IL-1 specifically, in inflammation and wound healing is clearly established. The foundations of our hypothesis on the role of IL-1 in human breast cancer is presented below.

IL-1 Cytokine Family

The IL-1 family consists of two polypeptides, IL-1α and IL-1β. These cytokines possess a wide spectrum of properties that encompass inflammatory, metabolic, physiologic, hemopoetic, and immunologic functions (21). Although the term interleukin means "between leukocytes," IL-1 is synthesized by and regulates a variety of cells, including leukocytes, macrophages, astrocytes, endothelial cells, smooth muscle cells, fibroblasts, synovial lining cells, dermal dendritic cells, keratinocytes, intestinal, gingival, and cervical epithelium, natural killer cells, and maternal placental cells (21). Because IL-1: 1) regulates cell function in a large variety of cells; 2) is present in many different organs; 3) is associated with numerous disease processes; and 4) can induce the expression of a variety of other cytokines and receptors, it is often considered a "primer", "inducer" or "controller cytokine" in the hierarchy of pro-inflammatory cytokines. IL-1α and IL-1β bind to two IL-1 receptor s, an 80-Kd receptor (IL-1 RtI), and a 68-Kd receptor (IL-1 RtII) (21). In general, IL-1α binds preferentially to the type I receptor, and IL-1β binds optimally to the type II receptor (22). A considerable volume of literature exists demonstrating the *in vitro* ability of IL-1 to regulate a variety of cellular functions in tumor cells (10, 23-29). Surprisingly little information exists on the *in vivo* expression and distribution of IL-1 in human cancers (30, 31).

IL-1 Family and Cancer

In vitro studies have shown that IL-1 can induce a variety of factors and functions in breast cancer cells. For example, malignant human breast tumors are known to contain high levels of prostaglandins. In a recent study (32), IL-1β induced PGE2 production in breast fibroblasts. In that study, only two BCC lines, MDA-MB-231 and Hs578T demonstrated increases of PGE2 in response to IL-1β. Several other BCC lines did not respond. IL-1 is also known to inhibit the growth of cultured BCC. For example, insulin and insulin like growth factor I (IGF-I) induced BCC proliferation was inhibited by the presence of IL-1α and IL-1β in MCF-7 cells. In that study, insulin receptor protein and mRNA were increased in the presence of IL-1β. Additional data in that study suggests that IL-1 antagonizes insulin and IGF-I mitogenic effects in MCF-7 by blocking tyrosine kinase (33). Danforth *et al*, examined the ability of IL-1 and IL-6 to inhibit BCC growth *in vitro*. These investigators found that both IL-1 and IL-6 inhibited growth of MCF-7 BCC. IL-1 alone had a greater effect than IL-6 alone. When the two were combined, the effect was synergistic. Further, IL-1 and IL-6 decreased the estradiol stimulated growth of the BCC. Additionally, their studies also demonstrated that IL-1, but not IL-6, caused increased secretion of TGF-β by the BCC (34).

Interestingly, studies by Speiser *et al* found that IL-1 up-regulated HLA class I and HLA Class II (DR) antigen expression on the cell surface of ZR-75-1 cells. This up-regulation of antigens was associated with increased TNF expression (35). Thus, IL-1 appears to play an important distribution in the regulation of breast cancer cell function and growth *in vitro*. Surprisingly, nothing is known about the presence and role of IL-1 in human breast cancer *in vivo*. Our present study is intended to fill this

gap in our knowledge regarding the presence and localization of IL-1 in human breast cancer.

In order to determine the distribution of IL-1, immunohistochemical analysis of breast cancer and non malignant breast specimens was performed, and demonstrated the presence of IL-1α and IL-1β associated with a variety of cells in the tumor microenvironment (see Table 1 and 3). Specifically, IL-1 cytokines were found to be associated with breast tumor epithelial cells, and with blood vessels found in the tumors. Our studies showed that IL-1α was found in endothelial cells associated small blood vessel present in HBC specimens. IL-1α expression was lower in the endothelial cells of large blood vessels in IBC or DCIS (39%, 30%), when compard to the vascular endothelial cells (57%) present in non-malignant breast tissue. A similar staining pattern was seen for IL-1β in the malignant tissue. The most striking difference was the generally lower level of IL-1β staining in the specimens from non-malignant breast tissue, when compared to IL-1α staining (see Tables 1 and 3).

Cytokine quatitation demonstrated the presence of IL-1α and IL-1β in HBC tumor homogenates (17.65 ± 3.9 pg./mg TP and 12.02 ± 1.42 pg./mg TP, respectively) in approximately equal concentrations. Our studies therefore clearly demonstrate the presence and distribution of IL-1α and β in malignant and non malignant breast tissues. Since tumor cells occupy a major portion of the tissue mass within the TME, it is reasonable to conclude that tumor cells are a major source of intracellular and extracellular IL-1α and IL-1β. Based on these observations we have constructed a hypothetical model describing the role of IL-1 cytokines within the breast cancer microenvironment. In this model, we hypothesize that IL-1 is primarily expressed by

tumor cells. The IL-1 thus produced then acts in both an autocrine and paracrine fashion to 1) regulate tumor and tissue cell proliferation, and 2) control production of protumorigenic factors such as angiogenic factors, growth factors, metalloproteases, etc. Interestingly, and in support of our hypothesis, tissue levels of both IL-1α and IL-1β both correlated with tissue levels of IL-8, a known angiogenic factor. Therefore, future *in vivo* studies to evaluate our hypothetical model are needed to directly demonstrate the pivotal role that the IL-1 family of cytokines plays in regulating tumor growth and metastasis.

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FIGURE LEGEND

Figure 1: IL-1α and IL-1β immunohistochemical analysis of invasive DCIS and benign human breast disease tissue. A) invasive tumor cell expression of of IL-1α (20x) B) invasive tumor cell expression of of IL-1β (20x) C) tumor cell control D) DCIS tumor cell expression of of IL-1α (20x) E) DCIS tumor cell expression of of IL-1β (20x) F) DCIS tumor cell control G) benign DEC expression of IL-1α (20x) H) benign DEC expression of IL-1β (20x) I) benign DCIS control (20x) J) VEC expression of IL-1α (20x) K)) VEC expression of IL-1β (20x) L) VEC control (20x)

Table I: IL-1α Immunohistochemistry: Positivity

Tissue	Tumors	Ducts	Small Vessels Endo	Large Vessels Endo	Vascular Smooth Muscles	Fibroblasts
Invasive (n=32)	32/32 (100%)	9/9 (100%)	22/31 (71%)	11/28 (39%)	14/28 (50%)	14/31 (45%)
in situ (n=11)	10/10 (100%)	9/10 (90%)	6/9 (67%)	3/10 (30%)	6/10 (60%)	5/10 (50%)
Non malignant (n=7)	N/A	7/7 (100%)	5/7 (71%)	4/7 (57%)	5/7 (71%)	4/7 (57%)

TABLE II: Immunohistochemical Staining Intensity for IL-1α and IL-1β in Human Breast Disease Specimens.

	IL-1α	IL-1β
	Mean Values	
Tissue	Tumor Mean ± SEM	Tumor ± SEM
Invasive	2.5 ± 0.11	1.86 ± 0.20
DCIS	2.5 ± 0.22	2.11 ± 0.32
Benign	1.5 ± 0.36	0.75 ± 0.25
	p Value	
Invasive vs. Benign	0.002	0.04
DCIS vs. Benign	0.006	0.025
Invasive vs. DCIS	(Not Significant)	0.52

Table III: IL-1β Immunohistochemistry: Positivity

Tissue	Tumors	Ducts	Small Vessels Endo	Large Vessels Endo	Vascular Smooth Muscles	Fibroblasts
Invasive (n=26)	23/26 (88%)	14/18 (77%)	21/26 (81%)	4/25 (16%)	18/25 (72%)	10/24 (42%)
in situ (n=9)	7/9 (78%)	5/9 (56%)	4/9 (44%)	1/9 (11%)	3/9 (33%)	4/9 (44%)
Non malignant (n=7)	N/A	6/7 (86%)	4/7 (57%)	2/7 (29%)	3/7 (43%)	0/7 0%

Table IV: Correlations Between IL-1 and IL-8 Cytokines in Human Breast Cancer Tissue Homogenates.

	IL-1β	IL-8
IL-1α	0.16 (82)	0.01 (82)
IL-1β	X	0.01